Remarks

Status of the Claims

Claims 38-42, 49, 81-90, 98-100 are currently pending in this application. Claims 43-48 have been withdrawn. Claims 38, 49, 81, 84, and 100 are presently amended. New claims 101 and 102 have been added.

Support for the amendments to claims 38, 49, 81, 84, and 100 and for new claims 101 and 102 can be found throughout the specification, for example, in paragraphs 30-35, 108, 109, 115, and 125-133. Claims 49 and 84 have been further amended to recite the influenza nucleoprotein (NP) transcript, and support for this amendment can be found throughout the specification, for example, in Examples 4-8. Claims 49 and 84 have been further amended to remove recitation of "preventing."

No new matter has been added by the present Amendment. Applicant specifically reserves the right to pursue the subject matter of the canceled or amended claims in a related application. The present Amendment is introduced for the sole purpose of furthering prosecution. Applicant respectfully requests reexamination and reconsideration of the case in light of the present Amendments and the following remarks. Each of the rejections levied in the Office Action is addressed individually below.

Information Disclosure Statement

The Examiner has objected to the Information Disclosure Statement filed on December 18, 2006 on the grounds that the non-patent literature documents Tompkins *et al.*, Verma *et al.*, and Zhang *et al.* were damaged and were not able to be scanned into the file. Please find attached to this Response an Appendix containing each of these three references. Applicant requests that the Examiner consider these three references and update the record to indicate that they have been considered.

Obviousness-Type Double Patenting

The Examiner has levied a *provisional* obviousness-type double patenting rejection, asserting that claims 38-42, 49, 81-90, and 90-100 pending in the present application are not patentably distinct from claims 12, 22, and 24-27 of co-pending U.S. application U.S.S.N. 11/259,434.

Applicant respectfully refrains from commenting on this rejection until such time as it matures into an *actual* rejection. Given the amendments to the present claims, and the potentially changing nature of the claims in the cited applications, comment at this time would not be prudent.

Rejection under 35 U.S.C. § 112 for lack of written description

Claims 38-42, 49, 84-90, and 99 stand rejected by the Examiner under 35 U.S.C. § 112, first paragraph, for alleged lack of written description. The Examiner states that the specification does not adequately support claims drawn to an siRNA targeted to an influenza virus or transcript associated with an influenza virus for treating or preventing influenza infection. Applicants respectfully disagrees.

As an initial matter, Applicant submits that the Examiner made a typographical error and did not intend to include claims 38-42 in this rejection. As stated by the Examiner on page 6 of the Office Action, "claims 81-83, 99 and 100, drawn to inhibition of a target transcript of a respiratory virus in a mammalian subject [are] considered to have adequate description." Applicant notes that claims 38-42, just like claims 81-83, 99, and 100, are drawn to inhibition of a target transcript of a respiratory virus in a mammalian subject. Therefore, Applicant submits that the Examiner inadvertently included claims 38-42 in the present rejection, and presents the following arguments with the understanding that only claims 49, 84-90, and 99 are rejected. Applicant respectfully requests that the Examiner confirm that Applicant's understanding is correct.

Regarding claims 49, 84-90, and 99, the Examiner states that the rejection involves "the lack of nexus linking such a *broad genera of structures* (any siRNA targeted to any influenza virus or any transcript associated with influenza virus) with such a specific function of treating or

preventing influenza virus" (emphasis added, Office Action p.6). The Examiner states that the rejected claims do not recite any sequence identifiers or identify by name any particular influenza virus transcripts. Applicant does not agree with the Examiner's position, but is willing to amend the claims in accordance with her suggestion, with the hopes that such an amendment will further prosecution. Applicant, therefore, has amended independent claims 49 and 84 to specify that the influenza target transcript is an influenza nucleoprotein (NP) transcript. Applicant respectfully submits that the specification fully supports influenza or a clinical condition associated with overexpression or inappropriate expression of an influenza virus NP transcript or excessive functional activity of a polypeptide encoded by the NP transcript (see, for example, Examples 4-8 of the specification). Applicant, therefore, respectfully requests that the rejection be removed.

Rejection under 35 U.S.C. § 112 for alleged lack of enablement

Claims 49, 84-90, and 99 stand rejected under 35 U.S.C. § 112 for alleged lack of enablement. The Examiner states that the claims do not provide enablement for a method for *prevention* of influenza virus or *prevention* of a clinical condition associated with overexpression or inappropriate expression of an influenza virus transcript. Applicant respectfully disagrees.

Specifically, it is Applicant's understanding that the Patent and Trademark Office routinely rejects claims containing the word "preventing" on the ground that "preventing" is an absolute term and cannot ever be demonstrated. Applicant respectfully points out that this position is inconsistent with the art, where the term "preventing" is routinely used to refer to prophylactic therapy. Applicant notes that nowhere in the rejection does the Examiner refer to or address the scope of teaching provided in the present specification and submits that, were such an analysis to be performed, the Examiner would find that in fact the present specification is fully enabling of "prevention" as that term is understood in the art.

Notwithstanding the forgoing, and solely in order to further prosecution, Applicant has amended claims 49 and 84 to remove recitation of the word "preventing." Applicant, therefore, respectfully submits that the rejection is rendered moot in light of the present Amendment.

Rejection under 35 U.S.C. § 102(e) for alleged lack of novelty

Claims 38-42, 49, 81-90, 98, and 99 stand rejected by the Examiner under 35 U.S.C. § 102(e) on the grounds that they are anticipated by Biegelman *et al.* (U.S. Patent Publication 2003/0148928). The Examiner states that Biegelman teaches methods of inhibiting a transcript associated with a respiratory disorder, methods of preventing or treating a respiratory disease associated with overexpression or inappropriate expression of any transcript, comprising administering an siRNA in combination with a cationic polymer.

While not agreeing with the Examiner's position, and solely in order to further prosecution, Applicant has amended claims 38, 49, 81, 84, and 100 to recite "at least one cationic peptide" instead of "cationic polymers and modified cationic polymers." Biegelman *et al.* disclose particular compounds characterized as having an enzymatic nucleic acid at one of the compound and a peptide at the other end of the molecule. Biegelman *et al.* do not make any mention of "cationic peptides," and certainly do not disclose that cationic peptides can function as delivery agents, as recited in the present claims. Applicant submits that Biegelman *et al.* do not teach all of the elements of the claims and, therefore, do not anticipate the claimed invention. Applicant respectfully requests that the rejection be removed.

Rejection under 35 U.S.C. § 103(a) as allegedly being obvious

Claims 38-42, 49, 81-90, and 98-100 stand rejected under 35 U.S.C. § 103(a) on the ground that they are unpatentable over Agrawal *et al.* (U.S. Patent 5,194,428), Tuschl *et al.* (PCT Publication WO 02/44321), Gautum *et al.* (2000, *Mol. Therap.*, 2:63-70), and Kircheis *et al.* (1997, *Gene Therap.*, 4:409-18). The Examiner states that it would have been obvious to use polyethyleneimine (PEI) for delivery of siRNA into cells.

Applicant does not agree with the Examiner's position, but respectfully submits that the rejection is rendered moot by the present amendment. The present claims, as amended, recite methods involving use of compositions comprising an RNAi-inducing entity and a delivery agent comprising at least one cationic peptide. In contrast, none of Tuschl *et al.*, Agrawal *et al.*, Gautum *et al.*, or Kircheis *et al.* make any mention of a cationic peptide. Considering that these references, alone or in combination, do not teach every element of the claims, they cannot render the claims obvious. Applicant, therefore, respectfully requests that the rejection be removed.

Conclusion

Applicant, therefore, respectfully submits that the present case is in condition for allowance. A Notice to that effect is respectfully requested.

If, at any time, it appears that a phone discussion would be helpful, the undersigned would greatly appreciate the opportunity to discuss such issues at the Examiner's convenience. The undersigned can be contacted at (617) 248-5175.

Please charge any fees that may be required for the processing of this Response, or credit any overpayments, to our Deposit Account No. 03-1721.

Respectfully submitted,

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APPENDIX

Protection against lethal influenza virus challenge by RNA interference *in vivo*

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Communicated by Herman N. Eisen, Massachusetts Institute of Technology, Cambridge, MA, April 13, 2004 (received for review March 18, 2003)

Influenza virus infection is responsible for hundreds of thousands of deaths annually. Current vaccination strategies and antiviral drugs provide limited protection; therefore, new strategies are needed. RNA interference is an effective means of suppressing virus replication *in vitro*. Here we demonstrate that treatment with small interfering RNAs (siRNAs) specific for highly conserved regions of the nucleoprotein or acidic polymerase inhibits influenza A virus replication *in vivo*. Delivery of these siRNAs significantly reduced lung virus titers in infected mice and protected animals from lethal challenge. This protection was specific and not mediated by an antiviral IFN response. Moreover, influenza-specific siRNA treatment was broadly effective and protected animals against lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. These results indicate that RNA interference is promising for control of influenza virus infection, as well as other viral infections.

Influenza virus infection is a major public health problem, causing millions of cases of severe illness and as many as 500,000 deaths each year worldwide (1). Although inactivated vaccines are 60–80% effective against matched influenza strains (2), vaccination coverage is a problem worldwide. Moreover, this strategy provides no protection against unexpected strains, outbreaks such as the H5 and H7 avian influenza outbreaks in Hong Kong in 1997 and The Netherlands and Southeast Asia in 2003–2004, or pandemics. Currently, antiviral drugs are the best defense against these outbreaks, but they too provide only partial protection (3). New therapies to treat ongoing influenza infection are urgently needed, as well as new vaccination strategies inducing broader immunity (1, 4, 5).

RNA interference (RNAi) is an emerging technology that specifically inhibits gene expression. Small interfering RNAs (siRNAs), mediators of RNAi, are short (21–26 nt), double-stranded RNA duplexes that inhibit gene expression by inducing sequence-specific degradation of homologous mRNA (6). Many studies have shown that siRNA can significantly suppress gene expression when delivered into mammalian cells *in vitro* (7, 8). These findings raised the possibility that RNAi could inhibit viral gene expression and protect cells from viral infection. Subsequently, a number of studies demonstrated inhibition of replication of RNA viruses *in vitro* by RNAi (9–11), including HIV (12, 13), polio virus (14), hepatitis C virus (15, 16), West Nile virus (17), and influenza virus (17, 18). Moreover, a number of groups demonstrated effective silencing of both transgene and endogenous gene expression *in vivo* (19–25). Here we extend these studies to an animal model of virus infection and disease. We show that administration of influenza-specific siRNAs can decrease lung virus titers and protect mice from lethal challenge with a variety of influenza A viruses, including potential pandemic subtypes H5 and H7. This inhibition of influenza virus replication is specific, requiring homology between the siRNAs and gene targets, and is not the result of IFN induction by double-stranded RNA.

Materials and Methods

Mice. Female, 4- to 6-week-old BALB/cAnNCR mice were purchased from Division of Cancer Treatment, National Cancer Institute, Frederick, MD. Mice were challenged between 7 and 9 weeks of age. The institutions' animal care and use committees approved all protocols for all animal experiments.

Viruses. Influenza virus strains used were A/Puerto Rico/8/34 (PR/8, H1N1) (5), A/Hong Kong/156/97 (HK/156, H5N1) (26), A/NL/219/03 (Netherlands/219, H7N7) (27, 28), and A/Hong Kong/1073/99 (HK/1073, H9N2) (29). Virus stocks were propagated in the allantoic cavity of embryonated hen eggs at 34°C for 48–72 h (PR/8) or 37°C for 24 h (other viruses). All experiments with H5H1, H7N7, and H9N2 viruses were conducted under BSL-3+ containment.

siRNAs. siRNAs were purchased from Dharmacon Research (Lafayette, CO) as dried, 2′-deprotected, desalted duplexes and resuspended in AccuGENE PBS (BioWhittaker). Sequences used were as follows: GFP-949 (siGFP), sense 5′-GGCUACGUCCAGGAGCGCAUU-3′, antisense 5′-UUCCGAUGCAGGUCCUCGCGU-3′; nucleoprotein (NP)-1496 (siNP), sense 5′-GGAUCUUAUUUCUUCGGAGdTdT-3′, antisense 5′-dTdTCCUAGAAUAAAGAAGCCUC-3′; and acidic polymerase (PA)-2087 (siPA), sense 5′-GCAAUUGAGGAGUGCCUGAdTdT-3′, antisense 5′-dTdTCGUUAACUCCUCACGGACU-3′ [as reported by Ge *et al.* (18)].

siRNA Delivery and Virus Infection *in Vivo*. On day −1, siGFP, siNP, siPA, or combined siNP and siPA were diluted to 50 μg/ml (25 μg/ml of each with siNP and siPA combined) in PBS. Mice received 1 ml of diluted siRNA (3.78 nmol) or PBS through hydrodynamic i.v. injection as described in ref. 19. Sixteen to 24 h later (day 0), siRNA/oligofectamine complexes were prepared: oligofectamine (Invitrogen) was diluted 1:1 in PBS and incubated for 10 min at room temperature (RT), and siRNAs were diluted to 1 mg/ml in PBS. Diluted oligofectamine and siRNA complexes (or PBS for vehicle controls) were combined in a 3:2 ratio and incubated for 20 min at RT. In all experiments except that detailed in Table 1, challenge viruses were diluted to a final volume of 10 μl per challenge dose in PBS. For each mouse, 10 μl of diluted virus was combined with 50 μl of oligofectamine/siRNA. Sixty microliters of virus/oligofectamine/siRNA (1.51 nmol siRNA) was administered intranasally (i.n.) under anesthesia with isoflurane (PR/8 virus; Table 1 and Fig. 1), CO_2 (PR/8 virus; Table 2 and Fig. 2), or ketamine/xylazine (1.98 and 0.198 mg per mouse, respectively; HK/156, NL/219, and HK/1073 viruses). For the experiment detailed in Table 1, viruses were diluted to a final volume of 50 μl per challenge dose in PBS. In this

Abbreviations: eID_{50}, egg 50% infective dose; i.n., intranasal(ly); NP, nucleoprotein; PA, acidic polymerase; RNAi, RNA interference; siRNA, small interfering RNA; $TCID_{50}$, tissue culture 50% infective dose.

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Table 1. Influenza A-specific siRNA treatment inhibits influenza A/H1N1 virus replication *in vivo*

Treatment*	*n*	Mean lung virus titer†	*P* value‡	Fold reduction§
siGFP	5	5.6 ± 0.4	—	—
siNP	5	3.8 ± 0.1	<0.0001	63
siPA	4	4.6 ± 0.1	<0.01	10
siNP+siPA	5	3.8 ± 0.2	<0.0001	63

—, not applicable.

*BALB/c mice were treated as indicated and challenged with 5×10^2 $TCID_{50}$ of PR/8. Two days later, animals were sacrificed and lungs were collected for virus titer.

†Expressed as $\log_{10}$ $TCID_{50}$/ml ± SEM.

‡One-way ANOVA statistical analysis on log-transformed data, followed with comparison with control (siGFP) by Dunnett's method.

§Compared with the siGFP-treated group.

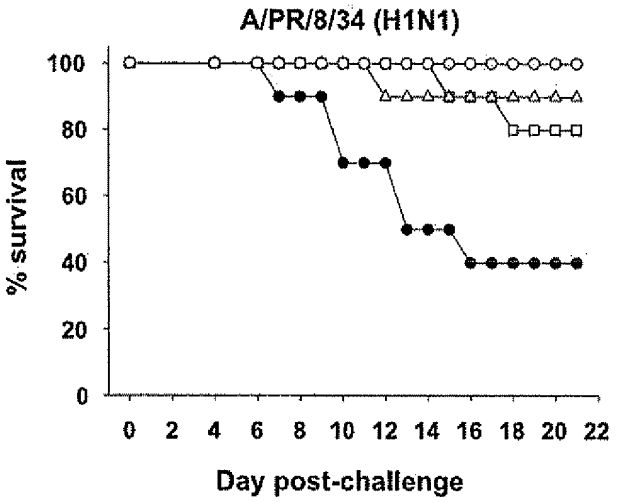

Fig. 1. Influenza-specific siRNA treatment protects mice from lethal H1N1 virus challenge. BALB/c mice (10 per group) were treated with siGFP (filled circles), siNP (open squares), siPA (open triangles), or siNP+siPA (open circles), challenged with PR/8 virus, and monitored daily for mortality. Influenza siRNA groups all differ from siGFP controls ($P < 0.05$; log-rank).

experiment, mice were anaesthetized with ketamine/xylazine, infected with the 50 μl PR/8 challenge dose i.n., and given the 50-μl i.n. siRNA dose (1.51 nmol) 20 min later while still under anesthesia. Virus challenge doses were as follows: 5×10^2 tissue culture 50% infective dose ($TCID_{50}$) of PR/8 virus, 10 LD_{50} of HK/156 virus, 10 LD_{50} of NL/219 virus, and 10^6 egg 50% infective dose (eID_{50}) of HK/1073 virus. Mice were killed day 2 postchallenge for analysis of lung virus titers or monitored for body weight and mortality until all animals had succumbed to infection or were recovering by body weight. Lungs for virus titer were homogenized in 3 ml of Leibovitz medium (Biofluids, Rockville, MD) and clarified by centrifugation. In the experiments detailed in Table 2, lung homogenates were titrated for virus infectivity by eID_{50} assay. For these assays, lungs were homogenized in 1 ml of sterile PBS and clarified by centrifugation.

Virus Quantitation. Madin–Darby canine kidney cells (MDCK) cells were cultured in OPTI-MEM I (Invitrogen). $TCID_{50}$ assays were performed as described in ref. 30. Briefly, lung homogenates or tissue culture supernatants were assayed for virus infectivity on MDCK by endpoint dilution for cytopathic effect with a 10-fold dilution series. Titers are expressed as $\log_{10}$ $TCID_{50}$/ml ± SEM. The detection limit of the assay is 1.5 $\log_{10}$ $TCID_{50}$/ml. For the lung titer experiment detailed in Table 2, lung homogenates were titrated by eID_{50} assay as described in ref. 5. Briefly, lung homogenates were titrated in 10-day-old embryonated eggs in 10-fold steps from initial dilutions of 1:10, and positive eggs were identified by hemagglutination with allantoic fluid. Values are expressed as $\log_{10}$ eID_{50}/ml ± SEM. The limit of virus detection is 1.2 $\log_{10}$ eID_{50}/ml.

Statistical Analysis. Lung virus titers were compared by statistical analysis on log-transformed data, using one-way ANOVA followed by Dunnett's test for treatment versus control or Student's *t* test. Results for percent initial body weight were compared by using Student's *t* test. Comparison of survival was done by using log-rank test.

Results and Discussion

We first confirmed the results of Ge *et al.* (18), showing that pretreatment of MDCK cells with siRNAs specific for NP 1496–1514 (siNP) or PA 2087–2106 (siPA) (18) could inhibit influenza replication after infection *in vitro*. siNP-, siPA-, or siNP+siPA-pretreated MDCK cells were infected with A/PR/8 (PR/8) virus. A GFP-specific siRNA, siGFP (18), was used in all experiments to control for potential nonspecific siRNA effects. Virus titers were decreased for at least 48 h postinfection compared with untreated or control siRNA-treated cells (data not shown).

Table 2. Influenza-specific siRNA treatment significantly decreases lung virus titers in mice challenged with H5, H7, and H9 avian influenza A viruses

Challenge virus*	Virus subtype	Treatment	Mean lung virus titer†	*P* value‡	Fold reduction§
PR/8	H1N1	siGFP	6.0 ± 0.2	—	—
		siNP + siPA	4.3 ± 0.6	0.0072	56
HK/156	H5N1	siGFP	7.6 ± 0.1	—	—
		siNP + siPA	6.5 ± 0.4	0.0201	11
NL/219	H7N7	siGFP	7.7 ± 0.4	—	—
		siNP + siPA	6.7 ± 0.2	0.0245	9
HK/1073	H9N2	siGFP	5.2 ± 0.2	—	—
		siNP + siPA	3.9 ± 0.6	0.0022	21

—, not applicable.

*BALB/c mice ($n = 4$) were treated and challenged as indicated. On day 2 postchallenge, animals were sacrificed and lungs were removed for virus titration by eID_{50}.

†Expressed as $\log_{10}$ eID_{50}/ml ± SEM.

‡One-way ANOVA statistical analysis on log-transformed data, followed by comparison using Student's *t* test.

§Compared with the siGFP-treated group.

To assess whether RNAi could inhibit influenza virus replication *in vivo*, we used an established murine model of influenza infection. BALB/c mice were treated with influenza-specific or control siRNAs by using hydrodynamic i.v. delivery as described by Lewis *et al.* (19). Sixteen to 24 h later, mice were infected with PR/8 i.n. and also given a second dose of siRNA in a lipid carrier i.n. in the hope of improving effectiveness in the lungs. Two days postchallenge, lungs were removed and lung homogenates were assayed for virus. Virus titers were significantly reduced in the lungs of animals given siNP, siPA, or siNP+siPA compared with those given siGFP (Table 1). Lung virus titers in untreated, PBS plus delivery vehicle-treated, and siGFP-treated animals were identical (data not shown), showing that siGFP treatment did not affect virus replication. Additionally, the decreases in virus titer were not due to effects in the assay of influenza-specific siRNAs in the lung homogenates; lung samples from siRNA-treated, unchallenged animals did not inhibit detection of virus-containing samples in the $TCID_{50}$ assay (data not shown).

Previous studies demonstrate that vaccines causing as little as 5- to 10-fold reduction in lung virus titers can protect against lethal influenza challenge (5, 30, 31). To determine whether the decreases in lung virus titers due to siRNA treatment were sufficient to protect animals from death, BALB/c mice were pretreated i.v. with siGFP, siNP, siPA, or siNP+siPA, followed 16–24 h later with lethal PR/8 challenge and a second i.n. dose of siRNA. By day 18 postchallenge (Fig. 1), 60% of animals given control siRNA had died, whereas mice given either siNP or siPA had significantly less mortality (20% and 10%, respectively). Strikingly, treatment with the combination of siNP+siPA resulted in 100% survival. In all cases, the protection provided by influenza-specific siRNA treatment was statistically significant.

Although a variety of studies testing virus-specific siRNAs *in vitro* found no induction of IFN-mediated antiviral responses (12, 14, 15, 18), it was recently reported by Sledz *et al.* (32) that siRNAs treatment could nonspecifically induce IFN-mediated innate immune responses. It was unlikely that IFN was mediating protection in our experiments, however, because animals treated with an identical amount of the control siGFP had significantly higher lung virus titers and significantly lower survival rates than animals treated with influenza-specific siRNAs (Table 1 and Fig. 1). Nonetheless, to verify that siNP+siPA was not inducing nonspecific antiviral responses, we tested serum and lung homogenates of mice treated with siRNAs in an IFN bioassay (33). We found no detectable IFN in an assay that readily detected IFN in the lungs of H3N2 virus-infected mice (data not shown). In addition, we compared the ability of the siRNA treatments to inhibit replication *in vivo* of PR/8 and influenza B/Ann Arbor/1/86 (B/AA). The influenza B/AA genome has only 52–67% homology with the siRNA target sequences. siNP+siPA treatment significantly decreased lung virus titers in PR/8-challenged animals, but not B/AA-challenged animals (data not shown), confirming that sequence homology between the siRNAs and the viral gene targets is necessary for suppression of virus replication.

Although influenza-specific RNAi provided potent protection against lethal challenge with PR/8, an H1N1 virus, it was unclear that it would protect against other influenza A subtypes because of the different kinetics and tissue tropism of these infections (34). The highly pathogenic avian influenza viruses that infected humans in the recent past were of particular interest because of their potential to unleash a pandemic; therefore, we tested the ability of siNP and siPA to inhibit replication of H5, H7, and H9 influenza subtypes. BALB/c mice were treated i.v. with siGFP or siNP+siPA on day −1. On the day of challenge (day 0), mice were given a second dose of siRNA in a lipid carrier i.n. and, at the same time, challenged with PR/8 virus (H1N1); HK/156 virus, an H5N1 isolate from the 1997 outbreak of avian influenza in

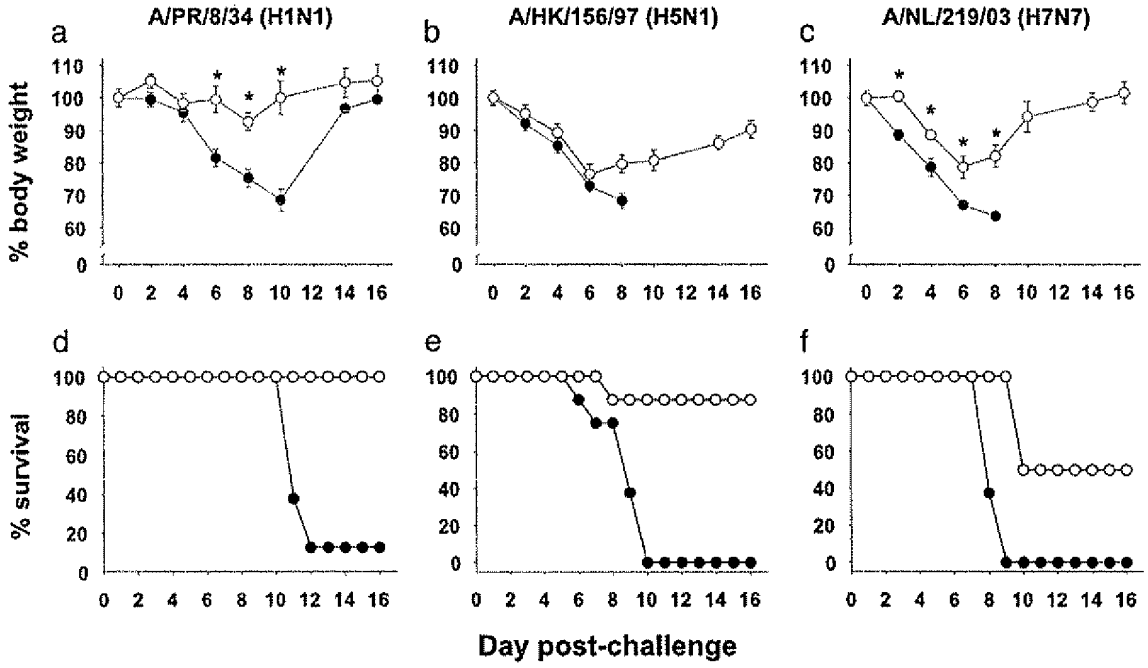

Fig. 2. Influenza-specific siRNA treatment is broadly cross-reactive and protects mice against lethal challenge with highly pathogenic H5 and H7 avian influenza A viruses. BALB/c mice (eight per group) were treated with siGFP (filled circles) or siNP+siPA (open circles) and challenged with PR/8 virus (*a* and *d*), HK/156 virus (*b* and *e*), or NL/219 virus (*c* and *f*). The percent of initial body weight (*a–c*) and survival postchallenge (*d–f*) are shown. Error bars (*a–c*) depict the standard error of the mean. *, Groups differ for weight loss, $P < 0.05$ (Student's *t* test). The single surviving mouse from the PR/8-challenged, siGFP-treated group recovered fully. siNP+siPA groups differ from siGFP groups for survival [$P < 0.002$ by log-rank analysis for virus strains in *d* (PR/8), *e* (HK/156), and *f* (NL/219)].

Hong Kong; NL/219 virus, an H7N7 isolate from the 2003 outbreak of avian influenza in The Netherlands; or HK/1073 virus, an H9N2 isolate from a 1999 case of avian influenza in Hong Kong. Two days later, animals were killed and lungs were tested for virus titer by eID_{50} assay. As shown in Table 2, lung titers from HK/156- and NL/219-challenged mice were reduced 11- and 9-fold, respectively, whereas lung titers from PR/8- and HK/1073-challenged mice were more dramatically reduced (56- and 21-fold, respectively; all reductions were statistically significant). Thus, the siNP+siPA treatment could suppress replication of a broad spectrum of influenza subtypes, including highly pathogenic avian isolates. Additionally, the siRNA-mediated inhibition of HK/156 replication provides further evidence that IFN is not responsible for inhibition of virus replication, because H5 viruses are resistant to the antiviral effects of IFNs (35).

Certain isolates of avian influenza that have infected humans cause infections in mice that are rapidly lethal at low challenge doses (34). To test whether the inhibition of virus replication by RNAi was adequate for protection, BALB/c mice were treated i.v. with siGFP or siNP+siPA and treated again 16–24 h later and given a lethal challenge i.n. with PR/8, HK/156, or NL/219 virus. We did not include HK/1073 virus, because it is minimally lethal in mice (T.M.T., unpublished data). Animals treated with siNP+siPA and challenged with a lethal dose of PR/8 survived, whereas almost 90% of the siGFP-treated mice died (Fig. 2 *a* and *d*). Survival results for H5N1 virus were dramatic; influenza-specific RNAi protected seven of eight mice challenged with a dose of HK/156 virus that killed all of the control mice (Fig. 2 *b* and *e*). In the case of NL/219, protection against mortality although partial, was significant at a challenge dose lethal to all of the siGFP controls (Fig. 2 *c* and *f*). In addition, morbidity as indicated by weight loss was significantly reduced for PR/8 and NL/219.

Influenza NP and PA proteins are essential to viral replication, providing ideal targets for RNAi (18). Moreover, the NP and PA genes are highly conserved across subtypes of influenza A virus; therefore, siRNAs against these genes should inhibit most influenza A viruses. This hypothesis is supported by our results demonstrating specific inhibition of replication of H1, H5, H7, and H9 influenza A subtypes *in vivo*. With the recent publication characterizing NL/219 by Fouchier *et al.* (28), the sequences of the NP and PA genes are known for all of the viruses tested. The siRNA target sequences were identical in PR/8, HK/156, HK1073, and NL/219. However, there are naturally occurring influenza variants that have mismatches in the targeted regions. It will be important to test the ability of these siRNAs to inhibit replication of viruses lacking complete identity. Targeting multiple elements within the influenza genome decreases the likelihood of mismatches in all RNAi targets and could reduce the likelihood of development of siRNA-resistant virus escape variants (14).

To use siRNA as an *in vivo* therapeutic, it must be delivered efficiently to the appropriate tissue(s). We demonstrate that hydrodynamic i.v. delivery combined with i.n. delivery of siRNA can specifically inhibit virus replication in the site of infection. Intravenous delivery of siRNAs alone also provided significant protection, although some animals succumbed to infection, suggesting that i.n. delivery contributed to survival (data not shown). Concurrent with our report, Ge *et al.* (36) show that i.n. delivery of plasmids expressing influenza-specific siRNAs can significantly decrease lung virus titers in influenza-infected mice. Studies are underway to test alternative expression vectors, delivery vehicles, and routes of administration.

Although we have not studied the effect of siRNA treatment of established infection, Ge *et al.* (36) have shown reduction of virus replication in the lungs by siRNA given after infection. Our studies demonstrate that siRNA can be effective when given to animals before an otherwise lethal influenza infection. Although it will be important to test siRNA treatment in established infections, the data reported here suggest that this intervention would be useful during influenza outbreaks, and that siRNA could be given as a preventive in the face of a pandemic. Further development of this technology may provide an effective strategy for controlling influenza and other viral diseases.

We are especially grateful to Dr. Edward A. Havell (North Carolina State University, Raleigh) for his rapid and thorough help with IFN measurement and analysis. Additionally, we are grateful to Roger Brock (U.S. Department of Agriculture/Agricultural Research Service/Southeast Poultry Research Laboratory, Athens, GA) for his assistance with BSL3+ experiments and Julia Misplon (Laboratory of Immunology and Developmental Biology, Center for Biologics Evaluation and Research/Food and Drug Administration, Bethesda) for technical assistance and many useful discussions. We thank Dr. Ron A. M. Fouchier (Department of Virology and National Influenza Center, Erasmus Medical Center, Rotterdam) for providing the sequences of NL/219 NP and PA genes before their availability in GenBank, Carolyn Wilson for critical review of the manuscript, and Andrew Byrnes for critical review of the manuscript and assistance with statistical analysis.

1. World Health Organization (2004) *Influenza: Report by the WHO Secretariat, World Health Assembly* (WHO, Geneva), A56/23 3-17-2003.
2. Wright, P. F. & Webster, R. G. (2001) in *Virology*, eds. Knipe, D. M., Howley, P. M., Griffin, D. E., Martin, M. A., Lamb, R. A., Roizman, B. & Straus, S. E. (Lippincott Williams & Wilkins, Philadelphia), pp. 1533–1579.
3. Nicholson, K. G., Aoki, F. Y., Osterhaus, A. E., Trottier, S., Carewicz, O., Mercier, C. H., Rode, A., Kinnersley, N. & Ward, P. (2000) *Lancet* **355**, 1845–1850.
4. Epstein, S. L. (2003) *Expert Rev. Anti-infective Ther.* **1**, 89–100.
5. Epstein, S. L., Tumpey, T. M., Misplon, J. A., Lo, C. Y., Cooper, L. A., Subbarao, K., Renshaw, M., Sambhara, S. & Katz, J. M. (2002) *Emerg. Infect. Dis.* **8**, 796–801.
6. Hannon, G. J. (2002) *Nature* **418**, 244–251.
7. McManus, M. T. & Sharp, P. A. (2002) *Nat. Rev. Genet.* **3**, 737–747.
8. Dykxhoorn, D. M., Novina, C. D. & Sharp, P. A. (2003) *Nat. Rev. Mol. Cell Biol.* **4**, 457–467.
9. Gitlin, L. & Andino, R. (2003) *J. Virol.* **77**, 7159–7165.
10. Haasnoot, P. C. J., Cupac, D. & Berkhout, B. (2003) *J. Biomed. Sci.* **10**, 607–616.
11. Silva, J. M., Hammond, S. M. & Hannon, G. J. (2002) *Trends Mol. Med.* **8**, 505–508.
12. Jacque, J. M., Triques, K. & Stevenson, M. (2002) *Nature* **418**, 435–438.
13. Lee, M. T., Coburn, G. A., McClure, M. O. & Cullen, B. R. (2003) *J. Virol.* **77**, 11964–11972.
14. Gitlin, L., Karelsky, S. & Andino, R. (2002) *Nature* **418**, 430–434.
15. Kapadia, S. B., Brideau-Andersen, A. & Chisari, F. V. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 2014–2018.
16. Randall, G., Grakoui, A. & Rice, C. M. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 235–240.
17. McCown, M., Diamond, M. S. & Pekosz, A. (2003) *Virol.* **313**, 514–524.
18. Ge, Q., McManus, M. T., Nguyen, T., Shen, C. H., Sharp, P. A., Eisen, H. N. & Chen, J. Z. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 2718–2723.
19. Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A. & Herweijer, H. (2002) *Nat. Genet.* **32**, 107–108.
20. McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J. & Kay, M. A. (2002) *Nature* **418**, 38–39.
21. Xia, H., Mao, Q., Paulson, H. L. & Davidson, B. L. (2002) *Nat. Biotechnol.* **20**, 1006–1010.
22. McCaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L. & Kay, M. A. (2003) *Nat. Biotechnol.* **21**, 639–644.
23. Giladi, H., Ketzinel-Gilad, M., Rivkin, L., Felig, Y., Nussbaum, O. & Galun, E. (2003) *Mol. Ther.* **8**, 769–776.
24. Song, E. W., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J. S., Shankar, P. & Lieberman, J. (2003) *Nat. Med.* **9**, 347–351.
25. Sorensen, D. R., Leirdal, M. & Sioud, M. (2003) *J. Mol. Biol.* **327**, 761–766.
26. Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., *et al.* (1998) *Science* **279**, 393–396.
27. Koopmans, M., Fouchier, R., Wilbrink, B., Meijer, A., Natrop, G., Osterhaus, A. D. M. E., van Steenbergen, J. E., du Ry van Beest Holle, M., Conyn van Spaendonck, M. A. E. & Bosman, A. (2003) *Eurosurveill. Wkly.* 7.

28. Fouchier, R. A., Schneeberger, P. M., Rozendaal, F. W., Broekman, J. M., Kemink, S. A., Munster, V., Kuiken, T., Rimmelzwaan, G. F., Schutten, M., Van Doornum, G. J., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 1356–1361.
29. Lu, X. H., Renshaw, M., Tumpey, T. M., Kelly, G. D., Hu-Primmer, J. & Katz, J. M. (2001) *J. Virol.* **75,** 4896–4901.
30. Benton, K. A., Misplon, J. A., Lo, C.-Y., Brutkiewicz, R. R., Prasad, S. A. & Epstein, S. L. (2001) *J. Immunol.* **166,** 7437–7445.
31. Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. (1994) *J. Immunol.* **152,** 1653–1661.
32. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H. & Williams, B. R. (2003) *Nat. Cell Biol.* **5,** 834–839.
33. Havell, E. A. (1986) *J. Infect. Dis.* **153,** 960–969.
34. Katz, J. M., Lu, X. H., Tumpey, T. M., Smith, C. B., Shaw, M. W. & Subbarao, K. (2000) *J. Virol.* **74,** 10807–10810.
35. Seo, S. H., Hoffmann, E. & Webster, R. G. (2002) *Nat. Med.* **8,** 950–954.
36. Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H. N. & Chen, J. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 8676–8681.

Advances in Brief

Small Interfering RNAs Directed against β-Catenin Inhibit the *in Vitro* and *in Vivo* Growth of Colon Cancer Cells[1]

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Abstract

The β-*catenin* and *APC* genes are key components of the Wnt signaling pathway. Mutation of these genes results in increased levels of the β-catenin protein, which is associated with enhanced cellular proliferation and the development of both colon polyps and colon cancer. Recently, a technique known as RNA interference has been successfully adapted to mammalian cells so that it is now possible to specifically decrease the expression of cellular genes after transfection of annealed small interfering 21-mer RNAs. In the current study, we used small interfering RNA (siRNA) directed against β-catenin to determine the effects of decreasing the high constitutive levels of this protein in colon cancer cell lines with mutations in either β-catenin or APC. Our studies demonstrate that siRNA directed against β-catenin markedly decreased β-catenin-dependent gene expression and inhibited cellular proliferation as reflected in the reduced growth of these colon cancer cells both in soft agar and in nude mice. These results indicate that siRNA can target specific factors whose expression is altered in malignancy and may have the potential as a therapeutic modality to treat human cancer.

Introduction

Colon cancer is one of the most common human malignancies, occurring in approximately 6% of the population in the United States (1). A frequent genetic abnormality seen in both hereditary and sporadic forms of polyps and cancer of the colon is mutation of the *APC* and β-*catenin* genes (2–8). These genes are key regulators of the Wnt pathway, which plays a critical role in the control of cellular proliferation.

The current model of Wnt signaling indicates that the binding of Wnt proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of the serine/threonine kinase GSK-3β.[4] GSK-3β is associated with β-catenin in a multiprotein complex that also includes the adenomatous polyposis coli tumor suppressor protein APC, axin or conductin, protein phosphatase 2A, and disheveled (5). GSK-3β phosphorylation of sites in the NH_2 terminus of β-catenin induces its degradation via the ubiquitin-proteasome pathway (9). After Wnt signaling, β-catenin associates with members of the TCF/lymphocyte-enhancer factor family and migrates to the nucleus, where this complex functions as a transcriptional activator (10). TCF/LEF in conjunction with β-catenin can activate the transcription of a variety of target genes including c-*myc* (11) and *cyclin D1* (12, 13).

A major regulator of β-catenin protein levels is APC (7, 14). Mutations in APC are frequently seen in both hereditary and sporadic colorectal cancers (6, 15), leading to the accumulation of β-catenin and increased levels of β-catenin/TCF-regulated transcription (4, 6, 8, 11). Some colon tumors that do not contain mutations in APC have increased levels of β-catenin as a result of mutations in the NH_2 terminus of β-catenin that prevent GSK-3β phosphorylation and subsequent degradation by ubiquitin-dependent proteolysis (9). Mutations in β-catenin and APC account for the majority of defects seen in tumors that have increased β-catenin levels (3–6).

Although the role of β-catenin in regulating cellular function has been extensively analyzed in other systems (2, 5), the ability to specifically reduce its levels by genetic means in established colon cancer cell lines is important for better defining its role in maintaining the malignant phenotype. Thus, we investigated whether specifically reducing the levels of β-catenin protein in established colon cancer cell lines, in which this protein was overexpressed, might result in decreased *in vitro* and *in vivo* proliferation. For this analysis, RNAi with siRNAs directed against β-catenin was used. RNAi is an evolutionary conserved mechanism that is operative in insects, nematodes, plants, and mammalian cells (16–18). In this process, sequence-specific posttranscriptional silencing is initiated by the introduction into cells of double-stranded annealed sense and antisense RNAs that are homologous to the sequence of the silenced gene (16). The ultimate mediators of RNAi-mediated degradation are 21-mer siRNAs that are generated by RNase III cleavage of double-stranded RNAs that have been introduced into cells and may extend up to several hundred nucleotides. For adaptation of

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[4] The abbreviations used are: GSK, glycogen synthase kinase; RNAi, RNA interference; TCF, T-cell factor; siRNA, small interfering RNA; NF-κB, nuclear factor κB; RSV, Rous sarcoma virus; TFIIB, transcription factor IIB; DEVD, Asp-Glu-Val-Asp; AMC, 7-amino-4-methylcoumarin; siRNAi, small interfering RNA interference; LEF, lymphoid enhancer factor; Tax, HTLV-I transactivator; CDK, cyclin dependent kinase; SPT, suppressor of transcription.

RNAi to mammalian cells, 21-mer sense and antisense RNA oligonucleotides that correspond to a portion of the gene of interest are synthesized and annealed (16–18). The annealed 21-mer RNAs are introduced into cells by transfection, where they bind specifically to the cellular mRNA of interest and activate a RNA degradation process that leads to 80–90% decreases in the corresponding protein levels. The use of 21-mer RNAs in mammalian cells, rather than longer RNAs that are used in other species, avoids the activation of the double-stranded dependent protein kinase, PKR, and nonspecific RNases that nonspecifically silence gene expression.

The studies reported here demonstrate that RNAi provides a simple, reproducible, and highly efficient means to determine the role of β-catenin on the growth of colon cancer cells both in culture and in nude mice. In addition, these data indicate that RNAi provides a useful methodology with which to study the role of regulatory genes that control the proliferation of cancer cells. Finally, our *in vivo* studies suggest that RNAi may have therapeutic potential in the treatment of cancer.

Materials and Methods

Cell Lines. The colon cancer adenocarcinoma cell lines SW480 (4, 14) and HCT116 (3, 4), with mutations in APC or β-catenin, respectively, were obtained from the American Type Culture Collection (Manassas, VA). These cells were propagated and maintained in McCoy's 5A and Leibovitz L-15 media (Life Technologies, Inc., Rockville, MD), respectively, and supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The HCT116 cells were maintained with 5% CO_2, whereas the SW480 cells were maintained without CO_2.

RNA Oligonucleotides. siRNA oligonucleotides with two thymidine residues (dTdT) at the 3′-end of the sequence were designed to β-catenin-1 (sense, 5′-AGCUGAUAUUGAUGGACAG-3′), which extends between amino acids 79 and 85 of β-catenin, β-catenin-2 (sense, 5′-CAGUUGUGGUUAAGCUCUUdAdC-3′), which extends between amino acids 491 and 498 of β-catenin, APC (sense, 5′-GCAACAGAAGCAGAGAGGU-3′), which extends between amino acid 238 and 245 of APC, the NF-κB p65 subunit (sense, 5′-GCCCUAUCCCUUUACGUCA-3′), which extends between amino acids 347 and 353 of p65; and HTLV-1 Tax (sense, 5′-GAUGGACGCGUUAUCGGCU-3′), which extends between amino acids 60 and 66 of Tax, along with their corresponding antisense oligonucleotides, as described previously (Ref. 18; Dharmacon Research Inc., Lafayette, CO). These RNAs were dissolved in TE [10 mM Tris-Cl (pH 8.0) and 1 mM EDTA] as 200 μM solutions. Double-stranded siRNAs were generated by mixing the corresponding pair of sense and antisense RNA oligonucleotides at 20 μM concentration in annealing buffer [30 mM HEPES-KOH (pH 7.9), 100 mM potassium acetate, and 2 mM magnesium acetate]. The reaction mixture was heated to 95°C for 2 min, allowed to come to room temperature over 30 min, and then aliquoted and stored at −20°C.

Transfection of RNA Oligonucleotides. Approximately, 1×10^6 cells were plated per 6-well plate in media containing 10% fetal bovine serum to give 30–50% confluence, and transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen, Carlsbad, CA) to result in a final RNA concentration of 50 nM. The cells were harvested at different time points and lysed in PD buffer [40 mM Tris-HCl (pH 8.0), 500 mM sodium chloride, 0.1% NP40, 6 mM EDTA, 6 mM EGTA, 10% glycerol, 10 mM sodium fluoride, and 1 mM sodium orthovanadate] for Western blot analysis.

To determine the effects of siRNA on β-catenin reporter constructs, the cells were transfected using 4 μl of GeneJuice (Novagen, Madison, WI) at 24 h after siRNA transfection with either TOPFLASH or FOPFLASH (0.5 μg) luciferase and a RSV-β-galactosidase reporter (0.1 μg; Ref. 8). The TOPFLASH and FOPFLASH reporters contain three wild-type or mutant β-catenin and TCF/LEF binding sites respectively inserted upstream of a minimal c-*fos* promoter. The cells were harvested after an additional 24 h, lysed in buffer (Promega, Madison, WI), and analyzed for luciferase and β-galactosidase activity using specific assays (Promega).

Western Blot Analysis. Cells were prepared in PD buffer, and Western blot analysis was performed as described previously (19). The antibodies and dilutions used included anti-APC (Ab-1; 1:1000; Oncogene, San Diego, CA), anti-β-catenin (1:1000; Transduction Laboratories, San Diego, CA), anti-c-*myc* (9E-10; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin D1 (1:500; Santa Cruz Biotechnology), anti-actin (1:1000; Santa Cruz Biotechnology), anti-CDK9 (1:1000; Santa Cruz Biotechnology), anti-p65 (1:1000; Santa Cruz Biotechnology), anti-SPT5 (1:1000; Ref. 19), or anti-TFIIB (1:1000; Transduction Laboratories). After extensive washing, the membranes were incubated with antimouse or antirabbit IgG-horseradish peroxidase conjugate antibody (Amersham, Piscataway, NJ) at a 1:2000 dilution for 1 h at room temperature and developed using enhanced chemiluminescence (Amersham).

Immunofluorescence Microscopy. HCT116 cells were grown on coverslips to 30–40% confluence and transfected with the various RNA oligonucleotides. At 72 h, the cells were processed for immunofluorescence as described previously (20) and analyzed on a Zeiss Axioskop 2 microscope at ×63 magnification. Antibodies against β-catenin and lamin B (Santa Cruz Biotechnology) were used at a dilution of 1:200, whereas the respective FITC- or Rhodamine Red-X-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:400 dilutions.

Cellular Proliferation Assays. HCT116 and SW480 cells transfected with the indicated RNA oligonucleotides were cultured in 96-cell well plates in replicates of 12. The cells were then incubated with 200 μl of tissue culture medium and pulsed with 1 μCi/well [^{3}H]thymidine (Perkin-Elmer, Boston, MA) for 12 h at 37°C. Extracts were prepared using a semiautomated cell harvester, and the [^{3}H]thymidine-labeled DNA was extracted and attached to glass fiber filter paper. The filter discs were air dried, transferred to a scintillation vial containing 3 ml of scintillation fluid, and counted on a Beckman LS 6000 liquid scintillation beta counter.

Caspase-3 Assays. Induction of apoptosis after siRNA transfection was assessed by detecting caspase activity in cell lysates 48 h after siRNA transfection. The activity of DEVD-specific proteases was measured using an EnzCheck Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) as suggested by the

manufacturer. At 48 h after siRNA transfection, the cells were harvested, and 1×10^6 cells were frozen at −80°C until the time of assay. The frozen cells were resuspended in 50 μl of lysis buffer and incubated on ice for 30 min, and cellular debris was pelleted. Lysates (50 μl) were transferred to 96-well plates and incubated at room temperature for 50 min with substrate (Z-DEVD-AMC) in 2× reaction buffer at a 100 μM final concentration. Cell lysate alone with the caspase inhibitor Ac-DEVD-CHO at a 100 μM final concentration was also included. Fluorescence was measured by a fluorometer at an excitation wavelength of 350 nm and a detection wavelength of 450 nm.

Soft Agar Colony Assays. At 24 h after siRNA transfection, the cells were mixed with tissue culture media containing 0.6% agar to result in a final agar concentration of 0.4%. Then 1 ml of this cell suspension was immediately plated in 6-well plates coated with 0.6% agar in tissue culture media (1 ml/well), and the colonies were counted 10 days after plating. The cultures were analyzed in triplicate, and the number of colonies/10^5 cells was calculated.

Murine Xenograft Model. Institutional guidelines and a Animal Research Committee-approved protocol were followed for mouse studies. For these studies, 4–6-week-old female nude$^{nu/nu}$ mice were obtained from Charles River (Wilmington, MA) and housed in clean specific pathogen-free rooms in groups of 5 and cages containing microisolator tops. At either 6 or 24 h after transfection of HCT116 cells with siRNA directed against either β-catenin (experimental group) or Tax (control group), the cells were harvested, washed twice in ice-cold serum-free McCoy's 5A media, counted for viable cells by trypan blue exclusion, and resuspended in the same media. The cells were resuspended in this same medium and used to inject each group of mice s.c. in the right flank with 2.5×10^6 cells in a volume of 0.25 ml. The tumors were measured in 3 axes from day 7 onward, and the tumor volume was calculated from these measurements. The survival was determined from the day of tumor injection to the day of euthanasia. As per institutional requirements, the mice were euthanized once tumors grew to greater than 2 cm.

In another experiment, 2×10^6 untreated HCT116 cells were injected into nude mice by i.p. injection. These mice were randomly divided into two groups of nine mice each. One group of mice was treated with siRNA directed against β-catenin, whereas the other group of mice was treated with siRNA directed against Tax. These mice were given i.p. injections containing 250 pmol of each of the siRNAs complexed with Oligofectamine and diluted into 0.5 ml of serum-free media. These treatments consisted of i.p. injections of the siRNAs administered once a day every 4 days for four doses starting 6 h after injection of the HCT116 cells. Afterward, the siRNAs were injected weekly for 3 additional weeks. Mice were carefully observed for survival, which was measured from day of injection of the HCT116 cells to either the day of spontaneous death or the day of euthanasia in the case of moribund mice.

Assessment of Expression β-Catenin in *in Vivo* Growing Tumor Samples. s.c. tumors were induced in nude mice by s.c. injection of 2.5×10^6 unmanipulated HCT116 cells, as described above. Seven days after induction of tumor, the mice were given 250 pmol of siRNA diluted in 250 μl of sterile PBS by i.v. injection in the lateral tail vein. The control group received Tax siRNA, whereas the experimental group was given β-catenin siRNA. Tissue samples were obtained 48–72 h after the i.v. dose of oligonucleotides, by core biopsy. The core biopsy was performed on tumors under anesthesia. Tissue samples were collected in PD buffer and incubated on ice for 30 min with intermittent mixing. After solubilization, the supernatant was collected by centrifugation at 14,000 rpm at 4°C for 20 min. The protein concentration was measured in each sample, and an equal amount of protein was used to analyze β-catenin levels by Western blot.

Statistical Analysis. Sigma Plot (SPSS Inc., Chicago, IL) was used to analyze the data and plot curves. Two-tailed unpaired *t* test was used to compare the statistical significance of the differences in data from two groups, where appropriate. Kaplan-Meier analysis was used to plot survival curves for mice injected with HCT116 cells after β-catenin or Tax siRNAi treatment.

Results

siRNA Directed against β-Catenin Reduces Its Expression in Colon Cancer Cell Lines with Mutations in Either APC or β-Catenin. In the majority of colon cancers, mutation in either the *APC* or β-*catenin* gene leads to increased levels of β-catenin (3, 4, 6, 8). The increased levels of β-catenin lead to the enhanced expression of β-catenin/TCF-regulated genes such as c-*myc* and *cyclin D1* (11–13), resulting in increased cellular proliferation (21–23). Two colon cancer cell lines, SW480 (4, 14) and HCT116 (3, 4), with mutations in either the *APC* or β-*catenin* gene, respectively, result in increased β-catenin levels (3, 4, 8, 14). In this study, RNAi was used in an attempt to decrease β-catenin expression in these cell lines to address whether its overexpression may have a role in regulating the proliferation of established colon cancer cells.

For these studies, annealed 21-mer sense and antisense RNA oligonucleotides directed against a portion of the β-*catenin* gene were used. In addition, RNA oligonucleotides directed against either the *APC* or the human T-cell leukemia virus *Tax* gene were used as controls. Each of these annealed RNAs was transfected into SW480 and HCT116, and their effects on β-catenin protein levels were compared with mock-transfected cells by Western blot analysis at 48 and 72 h posttransfection (Fig. 1*A*).

siRNA directed against β-catenin reduced the levels of β-catenin in both SW480 and HCT116 cells but did not affect the levels of actin (Fig. 1*A*). Interestingly, siRNA directed against β-catenin altered the mobility but not the level of APC, whereas siRNA directed against APC reduced the levels of this protein but had only minimal effects on the levels of β-catenin (Fig. 1*A*). SiRNA directed against Tax or mock transfection did not alter either β-catenin or APC levels (Fig. 1*A*). Similar results with β-catenin siRNAs were also seen in other cell lines including HeLa and 293 (data not shown). These results indicate that siRNA can markedly reduce β-catenin levels in colon cancer cell lines that have elevated levels of this protein as a consequence of mutations in either β-catenin or APC.

It was important to address whether siRNA directed against β-catenin altered either its level or cellular distribution when assayed by immunofluorescence. siRNA directed against either

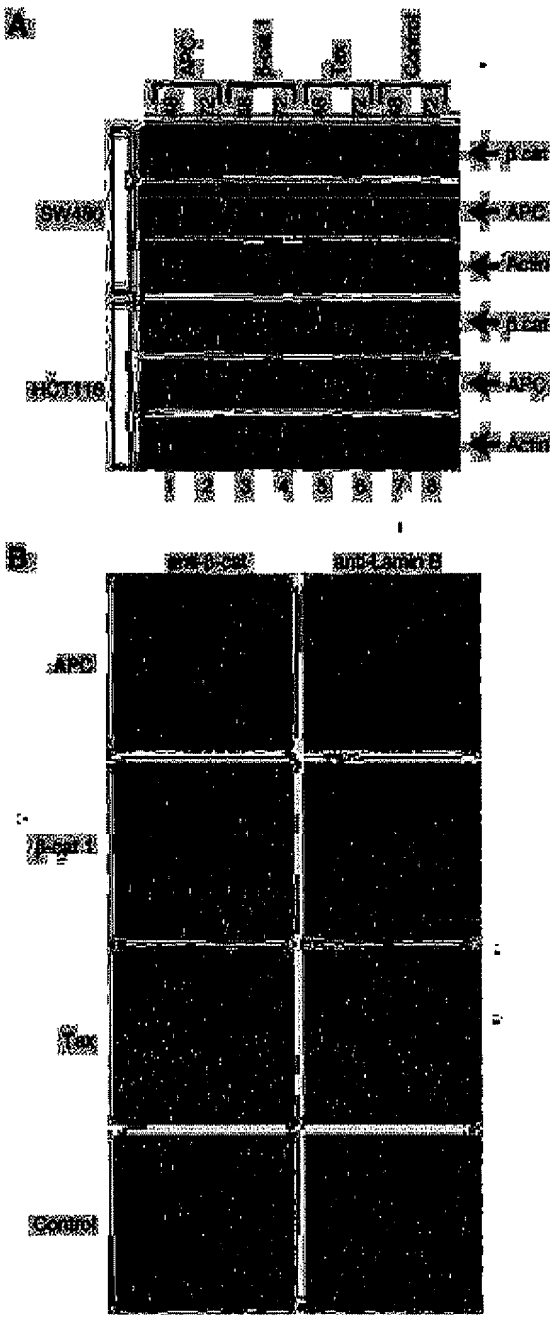

Fig. 1 siRNA directed against β-catenin specifically inhibits its expression. *A*, SW480 and HCT116 cells were transfected with 50 nM annealed sense and antisense 21-mer RNA oligonucleotides directed against either APC, β-catenin, or Tax containing the sequences listed in "Materials and Methods" or mock-transfected. Cells were harvested at either 48 or 72 h posttransfection, and Western blot analysis was performed. *B*, HCT116 cells grown on coverslips were transfected with the same annealed 21-mer RNA oligonucleotides used in *A* and stained with β-catenin or lamin B primary antibodies and Rhodamine Red-X- or FITC-conjugated secondary antibodies. The cells were then subjected to analysis with confocal microscopy.

APC, β-catenin, Tax, or mock transfection of HCT116 cells was performed, and these cells were then stained with antibody directed against either β-catenin or lamin B (Fig. 1*B*). In contrast to the membrane and nuclear staining of β-catenin that has been previously noted (20), siRNA directed against β-catenin reduced its levels such that it was not detected with an antibody directed against β-catenin (Fig. 1*B*). There was readily detectable nuclear staining of lamin B in these same cells. siRNA directed against APC resulted in slightly increased nuclear staining of β-catenin, whereas siRNA directed against Tax resulted in similar immunostaining of β-catenin as seen with control cells (Fig. 1*B*). Because APC has been demonstrated to enhance the nuclear export of β-catenin, siRNA-mediated decreases in APC can potentially result in increased nuclear levels of β-catenin and β-catenin-dependent gene expression (24). Thus, siRNA directed against β-catenin reduced its levels using both Western blot and immunofluorescence assays.

Time Course for siRNA-mediated Reductions in β-Catenin Protein. Next we addressed the duration of siRNA-mediated decreases in the expression of the β-catenin protein in both HCT116 and SW480 cells. In addition, we addressed whether siRNAs directed against another region of β-catenin could also reduce β-catenin levels and determined the specificity of β-catenin siRNA by assaying its effects on the expression of a variety of other cellular proteins. To address these points, siRNAs directed against either of two distinct regions of β-catenin (regions 1 and 2), APC, the p65 subunit of the NF-κB protein, or Tax were transfected into HCT116 and SW480 cells. The siRNA directed against the p65 NF-κB subunit was included in these studies to further assess the specificity of these siRNAs. Cells were harvested at 2, 3, 7, and 10 days after siRNA transfection, and extracts were prepared and analyzed by Western blot analysis for the expression of β-catenin and other transcriptional regulators including p65, SPT5, and CDK9, in addition to actin (Fig. 2).

This analysis revealed that β-catenin protein levels were reduced in both SW480 (Fig. 2*A*) and HCT116 (Fig. 2*B*) cells by siRNAs directed against two separate regions of β-catenin, but not by siRNAs directed against either Tax, APC, or p65 (Fig. 2). The levels of β-catenin were decreased at 48 h after siRNA transfection and remained reduced in both SW480 and HCT116 cells for 10 days after siRNA transfection (Fig. 2). A similar degree of inhibition over this time frame was seen with siRNA directed against the p65 subunit of NF-κB (Fig. 2). Western blot analysis demonstrated that the β-catenin siRNAs decreased β-catenin protein levels but not the levels of p65, SPT5, CDK9, or actin. Similarly, the p65 siRNA decreased the expression of p65 but not the levels of β-catenin, SPT5, CDK9, or actin. These results indicate that siRNAs have a high degree of specificity and resulted in prolonged decreases in specific cellular gene expression without marked effects on the expression of other cellular proteins.

β-Catenin-dependent Gene Expression Is Reduced by siRNA. When β-catenin is present in the nucleus, it is able to bind in conjunction with TCF/LEF to elements found in the promoters of a variety of cellular genes (10). A luciferase reporter with a minimal c-*fos* promoter and multiple binding sites for the TCF/LEF and β-catenin complex known as TOPFLASH and another luciferase reporter that contains mutated TCF/LEF binding sites

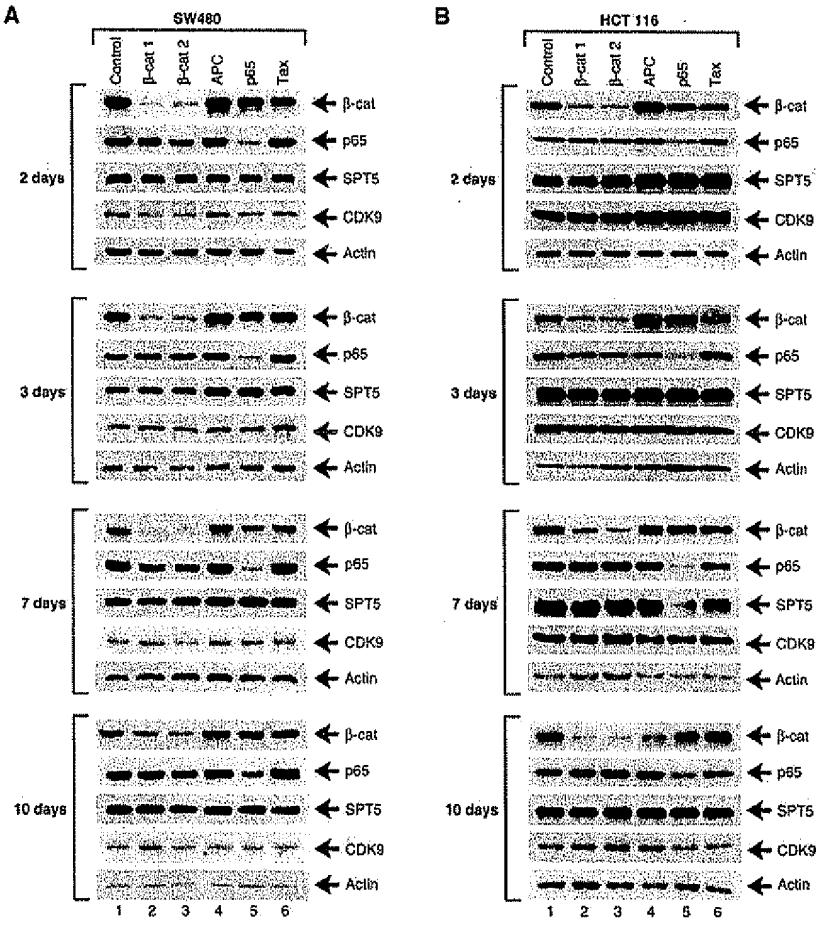

Fig. 2 Time course of siRNA reduction in β-catenin protein levels. (*A*) SW480 or (*B*) HCT116 cells were transfected with 21-mer annealed RNA oligonucleotides (50 nM) directed against either of two regions of β-catenin (1 and 2), the NF-κB p65 subunit, APC, or Tax or mock (control)-transfected. Extracts were prepared from each set of these cells at 2, 3, 7, or 10 days posttransfection and analyzed by Western blot analysis for the expression of β-catenin, p65, SPT5, CDK9, and actin.

known as FOPFLASH have been widely used to characterize β-catenin-dependent gene expression (8). These reporter constructs were transfected into siRNA-treated SW480 and HCT116 cells, and luciferase activity was determined.

siRNA directed against β-catenin, but not siRNA directed against APC or Tax, reduced TOPFLASH activity between 6- and 8-fold in SW480 cells (Fig. 3*A*). SiRNA directed against β-catenin also resulted in a 5–6-fold decrease in TOPFLASH activity in HCT116 cells (Fig. 3*B*). There was little effect of siRNA directed against β-catenin on the FOPFLASH reporter (Fig. 3, *C* and *D*). HCT116 cells contain a wild-type *APC* gene that can reduce the nuclear levels of β-catenin via its ability to export β-catenin to the cytoplasm (4). siRNA directed against APC markedly stimulated β-catenin-dependent gene expression in HCT116 cells (Fig. 3*B*). This result was consistent with the increased nuclear levels of β-catenin seen in these cells when analyzed by immunofluorescence (Fig. 1*B*). Finally, we addressed whether siRNA directed against β-catenin reduced the expression of two cellular genes known to be regulated by the TCF/LEF and β-catenin. The endogenous expression of c-*myc* and cyclin D1, but not the transcription factor TFIIB, was reduced by transfection of siRNA directed against β-catenin, but not APC or Tax (Fig. 3*E*). These results indicate that siRNA can specifically decrease the expression of β-catenin-dependent genes.

Role of β-Catenin on Regulating Cellular Proliferation. Previous studies have demonstrated that β-catenin increases cellular proliferation (22, 23), likely due to its ability to increase the expression of specific cellular genes (11–13). Increased β-catenin levels may play a role in the proliferative advantage seen in colon polyps and other premalignant lesions (5). Whether increased levels of β-catenin also regulate the proliferation of established cancer cell lines remains unclear.

First, SW480 and HCT116 cells were either mock-transfected or transfected with siRNA directed against either APC, β-catenin, or Tax. The number of SW480 (Fig. 4*A*) and HCT116 (Fig. 4*B*) cells was then determined at both 24 and 72 h posttransfection. These studies indicated that siRNA directed against β-catenin slightly but reproducibly decreased the growth of SW480 cells at 72 h posttransfection (Fig. 4*A*). There was a much greater effect of the siRNA directed against β-catenin on the growth of HCT116 cells, with a 40–50% reduction in the growth of these cells seen at 72 h posttransfection in four separate experiments (Fig. 4*B*). In addition, siRNAi directed against β-catenin was also used to determine its effects on [^{3}H]thymidine incorporation in both SW480 and HCT116 cells.

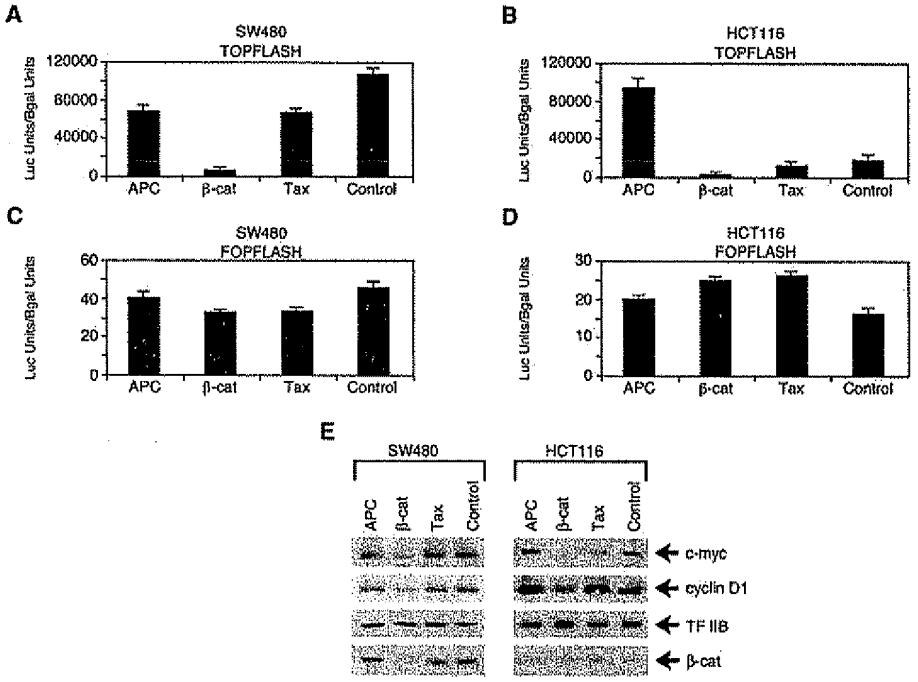

Fig. 3 siRNA directed against β-catenin reduces β-catenin-dependent gene expression. (*A* and *C*) SW480 and (*B* and *D*) HCT116 cells were transfected with 21-mer annealed RNA oligonucleotides (50 nM) directed against either APC, β-catenin, or Tax or mock-transfected. At 24 h posttransfection, the cells were transfected with either (*A* and *B*) TOPFLASH or (*C* and *D*) FOPFLASH reporters and a RSV β-galactosidase reporter. Luciferase and β-galactosidase activity were determined 48 h later. All transfections were normalized using RSV-β-galactosidase expression. The luciferase assays were performed in triplicate and repeated in three separate experiments. *E*, Western blot analysis was performed on extracts prepared from SW480 and HCT116 cells transfected for 72 h with RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected using antibodies directed against either β-catenin, c-myc, cyclin D1, or TFIIB.

RNAi directed against β-catenin resulted in a 20–30% decrease in the incorporation of [^{3}H]thymidine in both SW480 and HCT116 cells (Fig. 4, *C* and *D*). In contrast, RNAi directed against either APC or Tax did not result in significant decreases in [^{3}H]thymidine incorporation.

Finally, we wanted to address whether the decrease in growth or [^{3}H]thymidine incorporation seen in the β-catenin siRNA-transfected cells was due to increased levels of apoptosis. Caspase-3 activity in these siRNA-transfected cells was assayed in both the absence and presence of the caspase-3 inhibitor DEVD (Fig. 4, *E* and *F*). This analysis revealed that there was no increase in the level of apoptosis in either SW480 or HCT116 cells in the presence of the β-catenin siRNA. Western blot analysis of these samples confirmed that the β-catenin siRNAi resulted in decreased β-catenin levels (data not shown). These experiments, which were repeated on three separate occasions with similar results, suggested that β-catenin is important in regulating the proliferation of established colon cancer cell lines but does not lead to increased levels of apoptosis.

Reductions in β-Catenin Protein Inhibit Colony Formation. Given the effects of β-catenin siRNA on cellular proliferation, we next tested whether siRNA-mediated reductions in β-catenin levels decreased the ability of SW480 and HCT116 cells to form colonies in soft agar. To test this point, each of these cell lines was transfected with siRNA directed against either APC, β-catenin, or Tax or mock-transfected (Fig. 5, *A* and *B*). At 24 h posttransfection, the cells were placed into media with soft agar, and colony formation was assayed after 10 days. siRNA directed against β-catenin resulted in a significant decrease in colony formation in both SW480 (Fig. 5*A*) and HCT116 cells (Fig. 5*B*). There was a 3–4-fold decrease in colony formation seen in both SW480 and HCT116 cells transfected with siRNA directed against β-catenin, but not APC or Tax. These results suggested that reductions in β-catenin levels decreased the ability of colon cancer cells to form colonies in soft agar.

siRNA Directed against β-Catenin Reduces Tumor Growth *in Vivo*. Next, we addressed whether the transient exposure of HCT116 cells in culture to siRNA directed against β-catenin altered the ability of these cells to proliferate after injection into nude mice. In addition, we asked whether the *in vivo* administration of siRNA directed against β-catenin into nude mice after the i.p. injection of HCT116 cells could inhibit the growth of these cells and result in increased survival of these mice. Thus, we could address the potential *in vivo* effects of siRNA on inhibiting the proliferation of colon cancer cells.

In the first series of experiments, HCT116 cells were first transfected with siRNA directed against either β-catenin, Tax, or APC for either 6 or 24 h. Western blot analysis at 24 h posttransfection demonstrated a small reduction in β-catenin levels in the HCT116 cells transfected with β-catenin siRNA but not in those transfected with APC or Tax siRNAs (Fig. 6*A*). [^{3}H]Thymidine labeling of a portion of these HCT116 cells between 16 and 24 h after siRNA transfection revealed that there was little difference in the thymidine incorporation in these cells before their injection into nude mice (Fig. 6*B*). A similar number of these HCT116 cells that were transfected for either 6 (Fig. 6*C*) or 24 h (Fig. 6*D*) with siRNA directed against either β-catenin or Tax were then injected into two groups of five nude mice. At 4 weeks after injection of these siRNA-transfected HCT116 cells, the size of the tumors was determined.

As seen in Fig. 6, *C* and *D*, there was more than a 3-fold decrease in the average size of the HCT116 tumors derived from

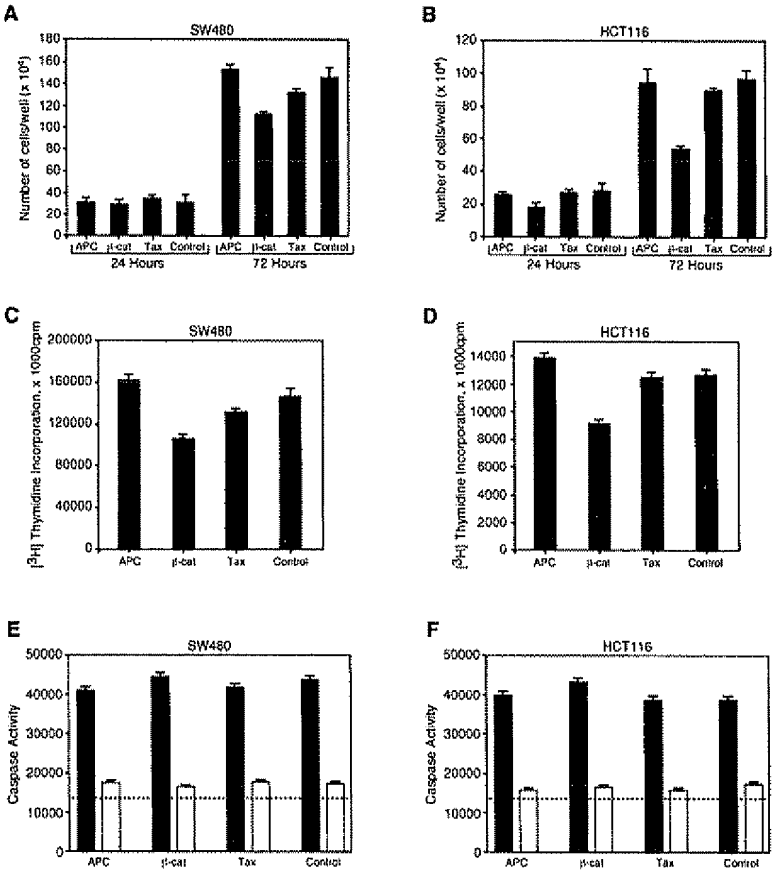

Fig. 4 siRNA directed against β-catenin leads to reduced cellular proliferation. (*A*, *C*, and *E*) SW480 and (*B*, *D*, and *E*) HCT116 cells were transfected with annealed 21-mer RNA oligonucleotides (50 nM) directed against either APC, β-catenin, or Tax or mock-transfected. *A* and *B*, after 24 h, the cells were trypsinized, replated in triplicate, and counted 24 and 72 h later by trypan blue exclusion with the SE indicated. *C* and *D*, HCT116 and SW480 cells were transfected with 50 nM concentrations of annealed 21-mer RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected (*Control*). At 60 h posttransfection, the cells were labeled with [^{3}H]thymidine for 12 h, and the amount of incorporation was determined for the same number of cells as described in "Materials and Methods." *E* and *F*, caspase-3 activity was assayed in HCT116 and SW480 cells transfected with siRNAs directed against APC, β-catenin, or Tax or mock-transfected. The activity of caspase-3 and related DEVD-specific proteases was assessed by fluoroscopic measurement of proteolytic cleavage of Z-DEVD-AMC to the fluorescent molecule AMC. The specificity of this reaction was assayed by measuring fluorescence in samples in the presence of the DEVD-specific protease inhibitor Ac-DEVD-CHO. Caspase activity in arbitrary units as measured by fluorescence of samples after incubation with substrate (■) and in presence of inhibitor (□) is shown. The *horizontal line* indicates baseline fluorescence.

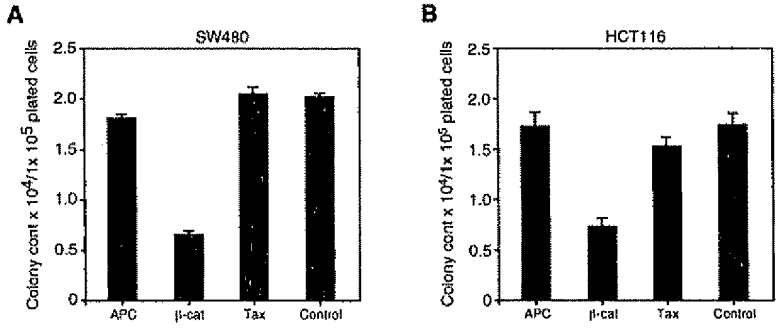

Fig. 5 siRNA directed against β-catenin reduces colony formation in soft agar. (*A*) SW480 and (*B*) HCT116 cells were transfected with annealed 21-mer RNA oligonucleotides (50 nM) directed against APC, β-catenin, or Tax or mock-transfected and plated in media containing soft agar at 24 h posttransfection. At 10 days posttransfection, the colonies were counted in three different plates, and the averages were plotted. The reduced number of colonies in the β-catenin group as compared with the control was statistically significant ($P < 0.01$).

cells transfected with siRNA directed against β-catenin as compared with HCT116 cells transfected with siRNA directed against Tax. These results were statistically significant with a P of <0.001 and <0.05, respectively. A Kaplan-Meier survival curve from an additional experiment using these siRNA-transfected HCT116 cells is shown in Fig. 6*E*. Of the five mice that received the HCT116 cells transfected with siRNA directed against β-catenin, two mice were still alive at 100 days after injection. In contrast, none of the mice who received the HCT116 cells transfected with siRNA directed against Tax were alive at this time.

Next, we addressed whether the i.p. injection of siRNAs directed against β-catenin as compared with Tax could alter the growth of HCT116 cells in nude mice (Fig. 6*F*). Approximately 2×10^6 HCT116 cells were injected i.p. into two groups of nine mice. These mice then received i.p. injection with 250 pmol of either Tax or β-catenin siRNAs, with the first injection performed 6 h after installation of the cells, and three additional injections were administered at 4-day intervals and then weekly for 3 weeks. There was one early death in the β-catenin siRNA group in the second week of the study due to unrelated causes because this mouse had no evidence of tumor at necropsy. Of the remaining mice, which were followed for a period of 70 days, none of the nine mice injected with Tax siRNA were alive,

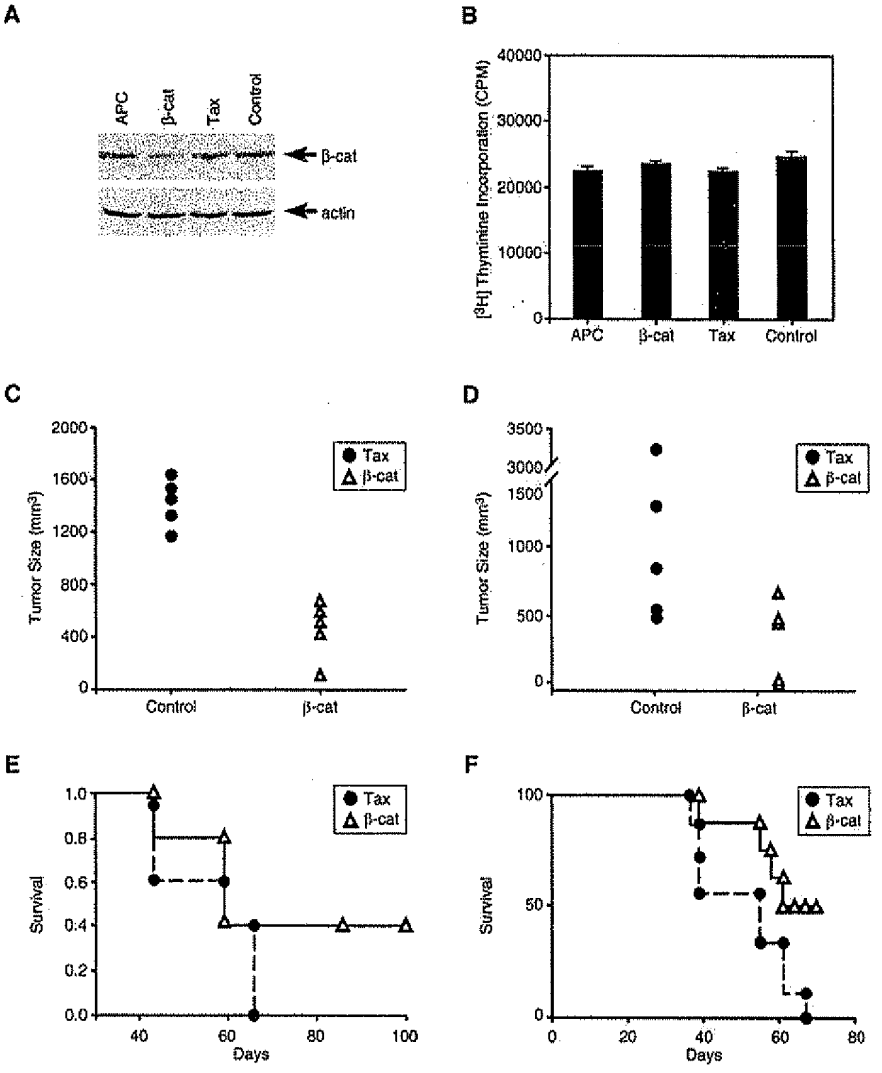

Fig. 6 siRNA directed against β-catenin reduces HCT116 tumor growth in nude mice. *A*, HCT116 cells were transfected with RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected. At 24 h posttransfection, extracts were prepared and analyzed by Western blot analysis using antibodies directed against β-catenin and actin. *B*, a portion of the HCT116 cells was also pulsed with [^{3}H]thymidine between 16 and 24 h after siRNA transfection, and [^{3}H]thymidine incorporation was determined. The HCT116 cells (2.5×10^6) used in *A* and *B* that were transfected with siRNAs directed against either Tax or β-catenin for either (*C*) 6 or (*D*) 24 h were injected into five nude mice each, as described in "Materials and Methods." Tumors were measured twice weekly, and their size at 4 weeks after injection is indicated. The *P* for difference between the growth of the tumors in the presence of Tax and β-catenin siRNA was $P < 0.05$ and $P < 0.001$, respectively. *E*, the survival of nude mice that received injection with 1×10^6 HCT116 cells transfected with siRNAs directed against either β-catenin or Tax was determined over a 100-day time period, and Kaplan-Meier analysis is shown. Mice were sacrificed when the tumor size reached 2.0 cm. *F*, two groups of nine nude mice each received i.p. injection with 2.5×10^6 HCT116 cells and then received i.p. injection with 250 pmol of siRNA directed against either β-catenin or Tax as described in "Materials and Methods." The survival of these mice was measured from the day of the initial tumor injection, and Kaplan-Meier survival probability was plotted. The survival of β-catenin siRNA-injected mice as compared with Tax siRNA-injected mice is shown ($P < 0.05$ by log-rank test).

whereas four of eight mice injected with β-catenin siRNA remained alive. Analysis of tumors from mice treated with either Tax or β-catenin siRNA revealed similar histology with no changes in the state of differentiation of the HCT116 cells. A Kaplan-Meier curve indicated that these survival differences were statistically significant with a $P < 0.05$ (Fig. 6*F*).

Finally, we addressed whether the administration of β-catenin siRNAs specifically reduced β-catenin levels after the i.v. administration of these siRNA. Two groups of five mice, each bearing HCT116 tumors that had grown for 1 week, received i.v. injection with 250 pmol of β-catenin or Tax siRNAs, and the tumors were biopsied 72 h later. The β-catenin siRNA significantly reduced β-catenin levels as compared with the Tax siRNA (Fig. 7). Thus, siRNA appears to be effective in reducing β-catenin levels and the *in vivo* growth of HCT116 cells in nude mice when administered by either transfection or after i.v. and i.p. injections.

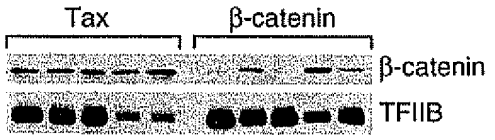

Fig. 7 Decreased expression of β-catenin in tumors of mice treated with siRNA to β-catenin. As outlined in "Materials and Methods," tumor samples were collected from mice treated with 250 mol of siRNA directed against either β-catenin or Tax, solubilized in PD buffer, and subjected to Western blot analysis. Immunoblots were analyzed using anti-β-catenin or anti-TFIIB (control) antibodies at 72 h postinjection.

Discussion

RNAi is an important technique with which to determine how decreases in the expression of specific regulatory genes alter the growth of both normal and cancer cells. In the current study, we determined how decreases in the high constitutive

levels of β-catenin in established colon cancer cells altered their *in vitro* and *in vivo* growth. Mutations in multiple components of the Wnt pathway including APC, β-catenin, and axin can result in increased levels of β-catenin (2, 5). The increased levels of β-catenin frequently found in both premalignant and malignant cells are associated with increased rates of cellular proliferation (5, 23). Moreover, modest overexpression of β-catenin in epithelial cells leads to increased proliferation and can result in transformation (21–23).

In our study, the functional consequences of RNAi-mediated decreases of β-catenin in established colon cancer cells were determined using assays of β-catenin-dependent gene expression, cellular proliferation, and *in vitro* and *in vivo* assays of tumor growth. In addition to mutations of β-*catenin* and *APC*, the cell lines used in this study have mutations in a variety of other cellular regulatory genes such as mismatch repair genes in the case of HCT116 and *p53* in the case of SW480 (25). Because mutations in the Wnt pathway leading to elevated levels of β-catenin frequently occur at an early stage of the neoplastic process, it was not clear whether reducing its expression at later stages of this process would decrease either cellular proliferation or tumor formation. Our analysis indicates that decreases in the levels of β-catenin in established colon cancer cell lines decrease proliferation by a mechanism that does not depend on increased apoptosis. Although it has been reported that down-regulation of signaling by β-catenin and TCF/LEF correlates with differentiation of Caco-2 cells in cell culture (26), histological analysis of the HCT116 xenografts did not demonstrate that siRNA directed against β-catenin resulted in increased differentiation of these cells.[5] These results suggest that reductions in TCF/LEF signaling may be associated with cell type-specific effects. In summary, our results demonstrated that reductions in β-catenin levels in established colon cancer cell lines decrease colony formation in soft agar and result in prolonged decreases in the growth of these cells when implanted into nude mice. These studies support a role for a β-catenin as an important factor in increasing the proliferation of established colon cancer cells and as a potential target to inhibit the growth of these cells.

Recent work suggests that after Wnt signaling, the dephosphorylated form of β-catenin is preferentially increased to result in higher levels of β-catenin-dependent gene expression (27). These data suggest that the β-catenin mutants in NH_2-terminal phosphorylation sites may have properties that differ from wild-type β-catenin. Targeted disruption of wild-type or mutant β-*catenin* genes in HCT116 cells was used to further characterize these alleles (28). This analysis revealed that the mutant β-catenin protein, as compared with the wild-type protein, exhibited decreased association with E-cadherin, had increased nuclear localization and transcriptional activity, and resulted in enhanced growth of cells when plated at low densities. However, disruption of the mutant β-catenin allele alone did not prevent the growth of HCT116 cells either in culture or in nude mice. Thus, disruption of the mutant β-*catenin* gene reduced the ability of HCT116 cells to form colonies in cell culture under specific conditions, although it was not essential for the growth of this established colon cancer cell line. It is important to note that in our studies, transfection of siRNAs reduced the amounts of both the wild-type and mutant β-catenin proteins and thus likely resulted in more severe decreases in β-catenin protein levels with greater effects on cellular proliferation than seen in targeted disruption of these individual alleles.

Because mutation of different components of the Wnt pathway including β-catenin, APC, axin, and protein phosphatase 2A can lead to β-catenin overexpression in cancer cells (3–6, 14), siRNA can be used to decrease β-catenin levels that result from mutations in different components of the Wnt pathway. For example, the elevated levels of β-catenin in SW480 cells resulting from mutations in the *APC* gene could also be targeted by siRNA directed against β-catenin. It was also interesting to note that siRNA directed against β-catenin resulted in alteration in the mobility of APC when analyzed by Western blot analysis. It seems likely that β-catenin binding to APC may either directly or indirectly alter APC phosphorylation or other posttranslational modifications as has been noted previously (29).

Antisense oligonucleotides have also been shown to inhibit β-catenin-dependent gene expression (30). Thus, questions arise about the relative efficacy of RNAi as compared with antisense oligonucleotides to decrease β-catenin-dependent gene expression. In this previous study, only 1 of 12 phosphorothioate oligonucleotides directed against β-catenin was able to decrease its protein expression by 80% (30). In contrast, siRNA targeting against multiple regions of β-catenin and a variety of other proteins demonstrated that approximately half of these siRNAs could result in 80–90% decreases in the level of this protein.[6] Furthermore, we found that a 2 μM concentration of phosphorothioate oligonucleotides was needed to significantly reduce the level of β-catenin, whereas the transfection of only 20 nM siRNAs directed against the same sequences in the β-*catenin* gene resulted in similar reductions in the level of this protein.[6] These results suggest potential advantages of siRNA as compared with phosphorothioate oligonucleotides for cell-based studies to reduce gene expression.

Our *in vivo* data suggest that siRNAs may have therapeutic potential for inhibiting the expression of genes that enhance the growth of tumors. Both transfection and *in vivo* administration of siRNAs directed against β-catenin led to reduced proliferation of HCT116 cells in nude mice. The ability of siRNAs directed against β-catenin to result in prolonged suppression of β-catenin levels in cell culture and reduce the *in vivo* growth of HCT116 cells when transfected for as little as 6 h suggests that RNA oligonucleotides are likely relatively stable in the cell. A variety of other genes such as HER-2/*neu*, c-*myc*, *bcl-2*, and specific components of the NF-κB pathway that are overexpressed in cancer cells may also be targeted by RNAi in an attempt to reduce their levels and thus decrease the proliferation of tumor cells (31). The ability to modify RNA oligonucleotides so that they are more stable *in vivo* will likely be necessary before adopting this technique for true *in vivo* therapy. In

[5] U. N. Verma and R. B. Gaynor, unpublished observations.

[6] R. M. Surabhi and R. B. Gaynor, unpublished observations.

summary, RNAi has been used in this study to demonstrate that reductions in β-catenin levels can reduce tumor growth under both *in vitro* and *in vivo* conditions. Given its specificity and the lower concentrations needed to inhibit gene expression as compared with those required for antisense oligonucleotides, RNAi may have potential therapeutic utility in a variety of disease states.

Acknowledgments

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References

1. Cancer statistics 2001. Mellors, R. C. Cancer statistics and predisposition to cancer. CA Cancer J. Clin., *51:* 15–20, 2001.

2. Hajra, K. M., and Fearon, E. R. Cadherin and catenin alterations in human cancer. Genes Chromosomes Cancer, *34:* 255–268, 2002.

3. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. β-Catenin mutations in cell lines established from human colorectal cancers. Proc. Natl. Acad. Sci. USA, *94:* 10330–10334, 1997.

4. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science (Wash. DC), *275:* 1787–1790, 1997.

5. Polakis, P. Wnt signaling and cancer. Genes Dev., *14:* 1837–1851, 2000.

6. Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. Cancer Res., *58:* 1130–1134, 1998.

7. Su, L. K., Vogelstein, B., and Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. Science (Wash. DC), *262:* 1734–1737, 1993.

8. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC−/− colon carcinoma. Science (Wash. DC), *275:* 1784–1787, 1997.

9. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. β-Catenin is a target for the ubiquitin-proteasome pathway. EMBO J., *16:* 3797–3804, 1997.

10. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. Functional interaction of β-catenin with the transcription factor LEF-1. Nature (Lond.), *382:* 638–642, 1996.

11. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Identification of c-MYC as a target of the APC pathway. Science (Wash. DC), *281:* 1509–1512, 1998.

12. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA, *96:* 5522–5527, 1999.

13. Tetsu, O., and McCormick, F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature (Lond.), *398:* 422–426, 1999.

14. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. USA, *92:* 3046–3050, 1995.

15. Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science (Wash. DC), *256:* 668–670, 1992.

16. Bass, B. L. Double-stranded RNA as a template for gene silencing. Cell, *101:* 235–238, 2000.

17. Caplen, N. J., Parrish, S., Imani, F., Fire, A., and Morgan, R. A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA, *98:* 9742–9747, 2001.

18. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature (Lond.), *411:* 494–498, 2001.

19. Ivanov, D., Kwak, Y. T., Guo, J., and Gaynor, R. B. Domains in the SPT5 protein that modulate its transcriptional regulatory properties. Mol. Cell. Biol., *20:* 2970–2983, 2000.

20. Lamberti, C., Lin, K. M., Yamamoto, Y., Verma, U., Verma, I. M., Byers, S., and Gaynor, R. B. Regulation of β-catenin function by the IκB kinases. J. Biol. Chem., *276:* 42276–42286, 2001.

21. Orford, K., Orford, C., and Byers, S. W. Exogenous expression of β catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest. J. Cell Biol., *146:* 855–867, 1999.

22. Zhu, A. J., and Watt, F. M. β-Catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development (Camb.), *126:* 2285–2298, 1999.

23. Morin, P. J., Vogelstein, B., and Kinzler, K. W. Apoptosis and APC in colorectal tumorigenesis. Proc. Natl. Acad. Sci. USA, *93:* 7950–7954, 1996.

24. Rosin-Arbesfeld, R., Townsley, F., and Blenz, M. The APC tumour suppressor has a nuclear export function. Nature (Lond.), *406:* 1009–1012, 2000.

25. Popanda, O., Zheng, C., Magdeburg, J. R., Buttner, J., Flohr, T., Hagmuller, E., and Thielmann, H. W. Mutation analysis of replicative genes encoding the large subunits of DNA polymerase α and replication factors A and C in human sporadic colorectal cancers. Int. J. Cancer, *86:* 318–324, 2000.

26. Mariadason, J. M., Bordonaro, M., Aslam, F., Shi, L., Kuraguchi, M., Velcich, A., and Augenlicht, L. H. Down-regulation of β-catenin TCF signaling is linked to colonic epithelial cell differentiation. Cancer Res., *61:* 3465–3471, 2001.

27. Staal, F. J., Noort Mv., M., Strous, G. J., and Clevers, H. C. Wnt signals are transmitted through N-terminally dephosphorylated β-catenin. EMBO Rep., *3:* 63–68, 2002.

28. Chan, T. A., Wang, Z., Dang, L. H., Vogelstein, B., and Kinzler, K. W. Targeted inactivation of CTNNB1 reveals unexpected effects of β-catenin mutation. Proc. Natl. Acad. Sci. USA, *99:* 8265–8270, 2002.

29. Rubinfeld, B., Tice, D. A., and Polakis, P. Axin-dependent phosphorylation of the adenomatous polyposis coli protein mediated by casein kinase 1 ϵ. J. Biol. Chem., *276:* 39037–39045, 2001.

30. Roh, H., Green, D. W., Boswell, C. B., Pippin, J. A., and Drebin, J. A. Suppression of β-catenin inhibits the neoplastic growth of APC-mutant colon cancer cells. Cancer Res., *61:* 6563–6568, 2001.

31. Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57–70, 2000.

nature medicine

Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene

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Respiratory syncytial virus (RSV) infection is one of the major causes of respiratory tract infection for which no vaccine or antiviral treatment is available. The RSV NS1 protein seems to antagonize the host interferon (IFN) response; however, its mechanism is unknown. Here, we used a plasmid-borne small interfering RNA targeting the NS1 gene (siNS1) to examine the role of NS1 in modulating RSV infection. RSV replication was reduced in A549 cells, but not IFN–deficient Vero cells, transfected with siNS1. siNS1 induced upregulated expression of IFN-β and IFN-inducible genes in A549 cells. siNS1-transfected human dendritic cells, upon RSV infection, produced elevated type-1 IFN and induced differentiation of naive CD4+ T cells to T helper type 1 (TH1) cells. Mice treated intranasally with siNS1 nanoparticles before or after infection with RSV showed substantially decreased virus titers in the lung and decreased inflammation and airway reactivity compared to controls. Thus, siNS1 nanoparticles may provide an effective inhibition of RSV infection in humans.

RSV is a major viral respiratory pathogen and produces an annual epidemic of respiratory illness causing bronchiolitis and otitis media in infants and young children[1,2] and pneumonia in adults and the elderly[3,4]. During 1991–1998, RSV was associated annually with over 17,000 deaths[5]. Immunodeficiency, cardiac arrhythmia and congenital heart disease are risk factors for more severe diseases with RSV infection[6–8]. An effective vaccine is not available for RSV and may not even be practical given the immunocompromised state of the target high-risk population, the incomplete immunity developed even by natural RSV infection and its short incubation period[9,10]. Consequently, DNA-based prophylactics are under investigation

RSV is the prototypic member of the genus *Pneumovirus* and is an enveloped, nonsegmented, negative-stranded RNA virus. The RSV genome of approximately 15,200 nucleotides is transcribed into 10 transcripts encoding 11 distinct proteins, including 2 nonstructural proteins, NS1 and NS2, which are expressed from separate mRNAs encoded by the first and second genes, respectively[11]. Deletion of either NS1 or NS2 severely attenuates RSV infection *in vivo* and *in vitro*, indicating that NS proteins have an important role in viral replication[12–15]. Furthermore, repeated RSV infections are common as a result of the incomplete immunity caused by natural infection, the basis of which is poorly understood[16]. RSV infection was shown to be associated with a predominantly T helper type 2 (T_H2)-like response in infants[17], although results of studies in children have been inconsistent. Hence, RSV is considered a predisposing factor for the development of allergic diseases and asthma[18,19].

IFNs attenuate RSV replication and also have therapeutic value against allergic diseases, including asthma[20–22]. We and others have developed *in vivo* intranasal gene delivery approaches using nanoparticles composed of chitosan, a natural, biocompatible and biodegradable polymer[21–24]. Because bovine and human RSV NS1 seem to antagonize the type-1 IFN-mediated antiviral response[25–28], we reasoned that blocking NS gene expression might attenuate RSV replication and provide an effective antiviral and immune enhancement therapy. The short interfering RNA (siRNA) approach has proven effective in silencing a number of genes of different viruses[29]. Here we used this approach to examine the potential and mechanism of siNS1 to inhibit RSV replication in cultured human epithelial cells, modulate immunity against RSV in human dendritic cells and attenuate RSV infection in mice. The results show that siNS1-mediated silencing of the NS1 gene substantially suppresses RSV replication and modulates host immunity to RSV infection compared with control groups.

RESULTS

siNS1 inhibition of recombiant RSV infection

Two different siRNA oligonucleotide sequences for RSV NS1, siNS1 and siNS1a, and control siRNAs against HPV18E7 (siE7) and influenza virus PB2 (siPB2) were designed and cloned into the pSMWZ-1 vector[30]. Analysis of EGFP expression in A549 cells cotransfected with pEGFP and siNS1, siNS1a, siE7 or siPB2 showed that none of the siRNAs silence the EGFP gene (data not shown). Immunoblotting results showed that pretransfection of A549 cells with siNS1, but not siE7, substantially reduced the expression of NS1 proteins (**Fig. 1a**) but not that of other viral proteins (data not shown) at 24 h after infection with a recombinant RSV expressing GFP (rgRSV)[31]. To test whether siNS1 attenuates

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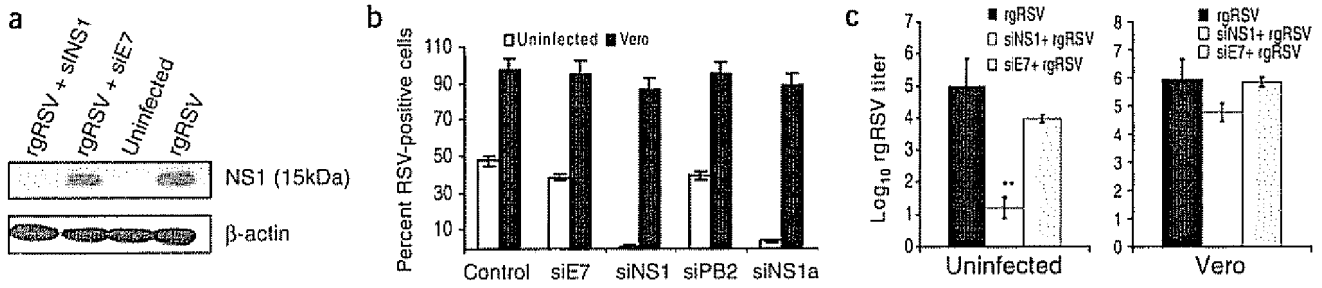

Figure 1 siNS1 inhibits rgRSV infection (a) Immunoblot analysis of NS1 protein expression at 24 h after infection with rgRSV (b) Flow cytometry analysis of rgRSV-positive uninfected cells and Vero cells, respectively Control versus siNS1 and control versus siNS1a, $P < 0.01$ (c) Measurement of virus titer using plaque assay Data are the averages of two independent experiments; $^{**}P < 0.01$ when compared with control group.

virus infection, we transfected A549 cells and type-1 IFN–deficient[32] Vero cells with the siNS1, siNS1a or control siRNAs, and then infected them with rgRSV. The results of flow cytometry showed a significant ($P < 0.01$) decrease in the percentage of cells expressing EGFP. In marked contrast to A549 cells, siNS1 and siNS1a did not decrease viral replication in Vero cells as compared to controls (Fig. 1b). Furthermore, plaque assays for RSV titers in culture supernatants indicated that siNS1 significantly decreases ($P < 0.01$) rgRSV titer compared to controls in A549 but not Vero cells (Fig. 1c). Plaque assays using siNS1a gave results similar to those from siNS1 (data not shown). Together, these results indicate that siNS1 attenuates RSV infection in a gene-specific fashion, and this attenuation may involve modulation of the type-1 IFN pathway by NS1

Mechanism of siNS1-mediated upregulation of the type-1 IFN pathway

The finding that RSV infection of A549, but not Vero, cells is affected by siNS treatment suggests a role of NS1 protein in the promotion of RSV infection by inhibiting the type-1 IFN pathway. To verify whether NS1 decreases type-1 IFN, we examined the expression of IFN-β by immunoblotting. The results show that A549 cells transfected with siNS1 or siNS1a, upon RSV infection, produce substantially increased amounts of IFN-β, compared to the different controls, including unrelated siRNA with no homology to mammalian genes (siUR) (Fig. 2 a,b). To further examine the role of NS1 in regulating the IFN pathway, we isolated RNAs from control and siNS1-transduced cells and subjected them to microarray analyses. The results show that siNS1 treatment increased the expression (≥6-fold change) of 25 IFN-inducible genes compared to rgRSV infection alone (Table 1), and we investigated the expression of a number of altered genes by western blotting. The results show that the phosphorylated STAT1 (Ser727), STAT1, IRF1, IRF3, ISGF-3γ and MxA proteins were upregulated after siNS1 inhibition (Fig. 2c). To determine whether NS1 affects STAT1 and IRF1 translocation in A549 cells, we infected transfected cells with wild-type RSV (MOI = 0.1), fixed them 3 h later, permeabilized them and stained them with appropriate antibody. Cells treated with siNS1 showed significantly higher nuclear localization of phosphorylated STAT1 and IRF1 compared to controls ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2d,e), suggesting that the NS1 protein blocks trafficking of these proteins into the nucleus

Silencing NS1 polarizes human DCs toward a T_H1-promoting phenotype

Monocytes isolated from human peripheral blood were cultured with requisite cytokines to test whether siNS1 expression affects RSV-infected dendritic cell (DC) activity. Thus, we measured the concentration of IFN-α and IFN-β in the supernatants from cultured, infected, monocyte-derived DCs transfected with siNS1 or control siRNA. The data show that siNS1 treatment induced a significantly higher ($P < 0.05$) production of both type-1 IFNs in infected DCs than the control siRNA (Fig. 3a). Furthermore, to assess the effect of siNS1-treated DCs on T-cell function, we cultured allogenic naive $CD4^+$

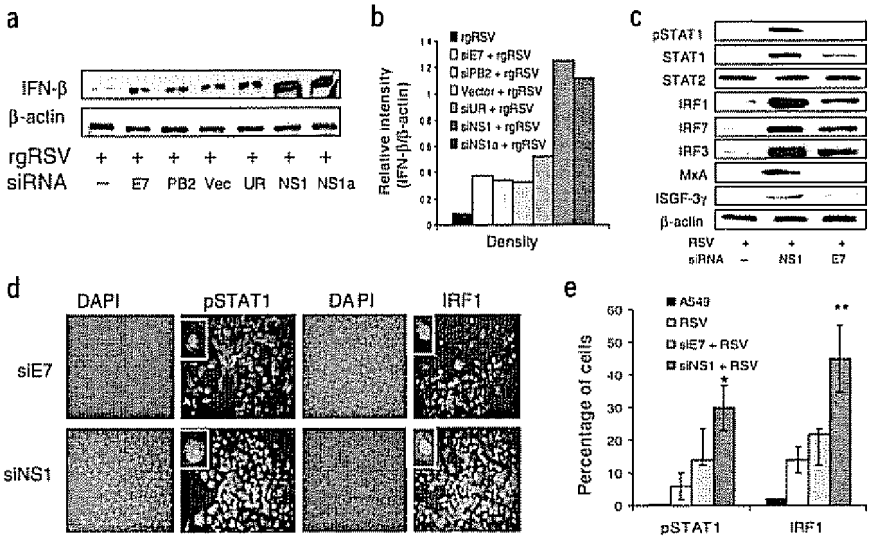

Figure 2 siNS1-mediated attenuation of RSV infection involves upregulated expression of IFN-β and IFN-inducible genes in infected A549 cells (a) Immunoblot analysis of IFN-β protein expression at 24 h after infection with rgRSV (b) Protein bands were scanned using the Scion image system (US National Institutes of Health) to quantify data in a (c) Immunoblot analysis of the expression of IFN-inducible genes 3 h after RSV-infected A549 cells For each, the results of one experiment of two performed with similar results are shown. (d) NS1 protein prevents nuclear import of IRF1 and STAT1 The nuclear localization of the IRF1 and STAT1 proteins in A549 cells was examined by indirect immunofluorescence using corresponding antibody and visualized and photographed under a fluorescent microscope (e) The percentage of positive cells was determined from 100 cells per field Data are mean ± s d from two separate experiments. $^*P < 0.05$ and $^{**}P < 0.01$ relative to control.

T cells with RSV-infected DCs treated with or without siNS1. The results of intracellular cytokine staining showed an increase in IFN-γ and a decrease in IL-4 secretion in naive $CD4^+$ T cells after they were cultured with siNS1-treated, RSV-infected DCs, compared with controls (Fig. 3b).

Nanoparticle-complexed siNS1(nano-siNS1) significantly attenuates RSV infection and pulmonary pathology in mice

To determine whether siNS1 exerts an antiviral response *in vivo* in BALB/c mice, we complexed the siNS1 plasmid (10 g per mouse) with a nanochitosan polymer (50 μg), referred to as Nanogene 042 (NG042). We administered the nanoparticles as a nasal drop 2 d before viral inoculation. NS1 expression in the lungs ($n = 6$) of mice was assayed by RT-PCR 18 h after infection. siNS1 substantially knocked down expression of the RSV NS1 gene but not the RSV F gene (Fig. 4a). The viral titer in supernatants of homogenized lungs ($n = 8$) was also significantly decreased in the siNS1 treated mice compared to controls ($P < 0.05$; Fig. 4b). We challenged these mice ($n = 8$) with methacholine at day 4 following rgRSV infection. RSV-infected mice showed a >400% increase in enhanced pause values compared to baseline and a 300% increase compared to the siNS1 group (Fig. 4c). Mice treated with siNS1 showed significantly lower ($P < 0.05$) AHR than that of untreated mice and showed a considerable reduction in pulmonary inflammation, as evidenced by decreases in the goblet cell hyperplasia of the bronchi and in the number of infiltrating inflammatory cells in the interstitial regions compared to controls (Fig. 4d). To assess IFN-β expression in the lung tissue of mice treated with siRNA 2d before viral inoculation, we extracted total RNAs from each group of animals, 24 h after infection ($n = 6$ per group) and assayed them by RT-PCR. Knockdown of the RSV NS1 gene significantly increased IFN-β expression in the lung compared to controls ($P < 0.05$; Fig. 4e,f). Examination of IFN-α levels in the bronchoalveolar lavage fluid by ELISA showed a twofold increase in IFN concentration in siNS1-treated mice compared to control mice (data not shown).

Table 1 IFN-inducible genes change more than sixfold in RSV-infected A549 cells

GenBank accession number	Gene	Function	Fold change (FC)[a]	Comparison[b] rgRSV	rgRSV + siNS1
NM_007315	STAT1	signal transducer and activator of transcription 1	6	D	I
NM_002198	IRF1	interferon regulatory factor 1	6	D	I
NM_001571	IRF3	interferon regulatory factor 3	6	NC	I
NM_004030	IRF7	interferon regulatory factor 7	6	D	I
NM_006084	IRF9	ISGF3G (p48)	6	D	I
NM_005531	IFI16	interferon gamma-inducible protein 16	6	D	I
NM_005532	IFI27	interferon, alpha-inducible protein 27	6	D	I
NM_006332	IFI30	interferon gamma-inducible protein 30	6	D	I
BF338947	IFITM2	interferon induced transmembrane protein 2	6	D	I
AL121994	1-8U	contains a pseudogene similar to IFITM3 (interferon induced transmembrane protein 3. STSs and GSSs	6	D	I
BE049439	IFI44	interferon-induced, hepatitis C-associated microtubular aggregate protein (44kD)	8	D	I
NM_004509	IFI41	SP110 nuclear body protein (interferon-induced protein 75, 52kD)	6	D	I
NM_003641	PTS	6-pyruvoyltetrahydropterin synthase- interferon induced transmembrane protein 1 (9-27) (IFITM1)	6	D	I
NM_005101	ISG15	interferon alpha-inducible protein (clone IFI-15K)	6	D	I
NM_002201	ISG20	interferon stimulated gene (20kD) (ISG20)	6	D	I
NM_022147	IFRG28	28kD interferon responsive protein	8	D	I
NM_002176	IFNB1	interferon beta 1, fibroblast	8	D	I
NM_002462	MxA	interferon-regulated resistance GTP-binding protein	6	D	I
NM_002463	MxB	interferon-regulated resistance GTP-binding protein	7	D	I
NM_016817	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	8	D	I
NM_003733	OASL	2'-5'-oligoadenylate synthetase-like	6	D	I
NM_016816	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	6	D	I
NM_006187	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	6	D	I
NM_001550	IFRD1	interferon-related developmental regulator 1	6	D	I
NM_001547	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	8	D	I

[a]Value for the fold change in expression calculated by the Microarray Suite 5.0 (MAS 5.0) program. [b]The data were compared to arrays of rgRSV-infected A549 cells either with or without siNS1 treatment. I, increased; NC, not changed; D, decreased.

Potential of Nano-siNS1 for prophylaxis and treatment of RSV infection

To investigate the persistence of siNS1 prophylaxis, we treated mice with the NG042-siNS1 complex at 2, 4 or 7 d before viral inoculation. Analysis of viral titers 5 d after infection showed that the prophylactic effect of siNS1 can last for at least 4 d, although treatment at day −7 still lowers viral titer by 1 $\log_{10}$ compared to the control (Fig. 5a). To test whether prophylactic blocking of NS1 activity can induce anti-RSV immunity and provide protection from reinfection, we administered the NG042-siNS1 complex to mice, inoculated them with RSV (5×10^6 plaque-forming units (p.f.u.)/mouse) 2 d later and then reinoculated them with RSV (1×10^7 p.f.u./mouse) after 16 d. Cellular immunity induced by RSV at 5 d after infection was examined in these mice by intracellular cytokine staining of splenocytes for IFN-γ and IL-4. Splenocytes of mice treated with NG042-siNS1 showed an increase in IFN-γ production in both $CD4^+$ and $CD8^+$ T cells and also increases in IL-4 production in $CD4^+$ T cells compared with controls (Fig. 5b,c). Examination of virus titer following secondary infection showed that mice treated with NG042-siNS1 showed a significant decrease in the viral titers compared to control mice ($P < 0.05$; Fig. 5d). Thus, prophylaxis with siNS1 enhanced cellular immunity and attenuated the secondary RSV infection.

To test the therapeutic potential of NG042-siNS1, we administered the NG042-siNS1 complex to mice at day 0 along with RSV inoculation or at day 2 or 3 after infection. Mice treated the same day as inoculation or at 2 d after RSV infection showed a significantly lower viral titer compared to controls ($P < 0.05$; Fig. 5e). Treatment with NG042-siNS1 3 days after inoculation also decreased virus titer, albeit marginally. Further, lung sections of mice treated with NG042-siNS1 2 days after RSV infection showed a substantial decrease in lung inflammation, goblet cell hyperplasia and infiltration of inflammatory cells compared to control mice (Fig. 5f).

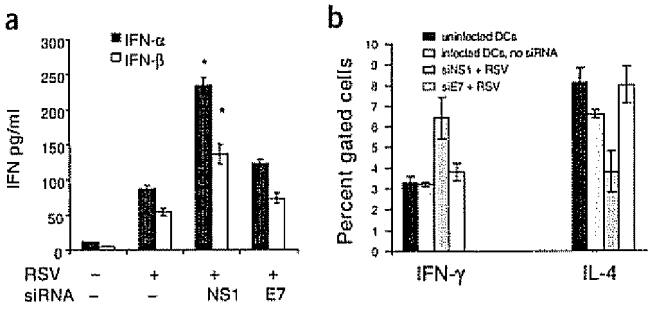

Figure 3 Effect of siNS1 on human DCs and naive CD4+ T cells (a) Expression levels of IFN-α and IFN-β protein in RSV-infected DCs, treated with or without siNS1 were measured by ELISA $P < 0.05$ for siNS1 versus siE7 (b) Flow cytometric analysis of intracellular cytokine production in allogenic naive CD4+ T cells after coculture with RSV-infected DCs, treated with or without siNS1 Results shown are from one representative experiment of three repeats

DISCUSSION

Although the human RSV NS1 protein has type-1 IFN–antagonistic effects, the mechanism remains unknown This report underscores the substantial role of NS1 in RSV replication and immunity to RSV infection These studies show that the NS1 protein downregulates the IFN-signaling system by deactivation of STAT1, IRF1 and IFN-regulated gene expression, which are critical to suppressing IFN action Furthermore, the results show the potential for nanoparticles encapsulating siNS1 for the prophylaxis and treatment of RSV infections

Vector-driven *de novo* expression of siRNA to attenuate RSV infection has not yet been reported, although antisense oligonucleotide–mediated attenuation of RSV infection in African green monkeys has been reported[33]. The potential of this approach remains uncertain as the effects of these oligonucleotides were measured at the very early stage of infection (*i e*, 30 min after RSV challenge) Mechanistically, both antisense and siRNA work post-transcriptionally to reduce expression of the target gene The antisense oligonucleotides accumulate in the nucleus and may alter splicing of precursor mRNA[34,35] In contrast, siRNAs exert their effects in the cytoplasm[36], which is the site of RSV replication Also, intracellular expression from RNA polymerase III promoters enables the production of stably expressed siRNA in the cell with sustained knockdown of the target, and hence, lower concentrations are needed to achieve levels of knockdown that are comparable to those from antisense reagents

We demonstrate in this report that DNA-vector driven siNS1 expression is capable of considerably attenuating RSV infection of human epithelial cells, which are the primary targets of RSV replication We used A549 epithelial cells, as they are similar to cultured primary airway cells in terms of their susceptibility to RSV[37]. The transfection efficiency of the construct as assessed using plasmid pEGFP was 43 21% and 49 62% in A549 and Vero cells, respectively Despite this, the siNS1 plasmid inhibited rgRSV production by 90–97%, which is consistent with a 2- to 3-$\log10_{10}$ decrease in RSV titers. Furthermore, two different siRNA constructs targeting NS1 showed almost identical results Although the mechanism of the siNS1-mediated decrease in viral titers was not investigated, it may be attributed to the fact that NS1, located at the 3′ end of the viral genome, acts as a common early-stage promoter for the initiation of both replication and transcription[38]. These results are consistent with reports that suggest that deletion of NS1 strongly attenuates RSV infection *in vivo* [12,14,15] and suggest the potential application of siNS1 for prophylaxis against RSV infection

We investigated the mechanism of siNS1-induced attenuation of viral replication To establish that the antiviral effects of siNS1 are the result of the modulation of the IFN pathway, we used Vero cells that lack the type-1 IFN genes and compared them with A549 cells Whereas A549 cells showed considerable siNS1- or siNS1a-induced decreases in rgRSV-infected cell numbers and virus titers, we saw no effect of siNS1

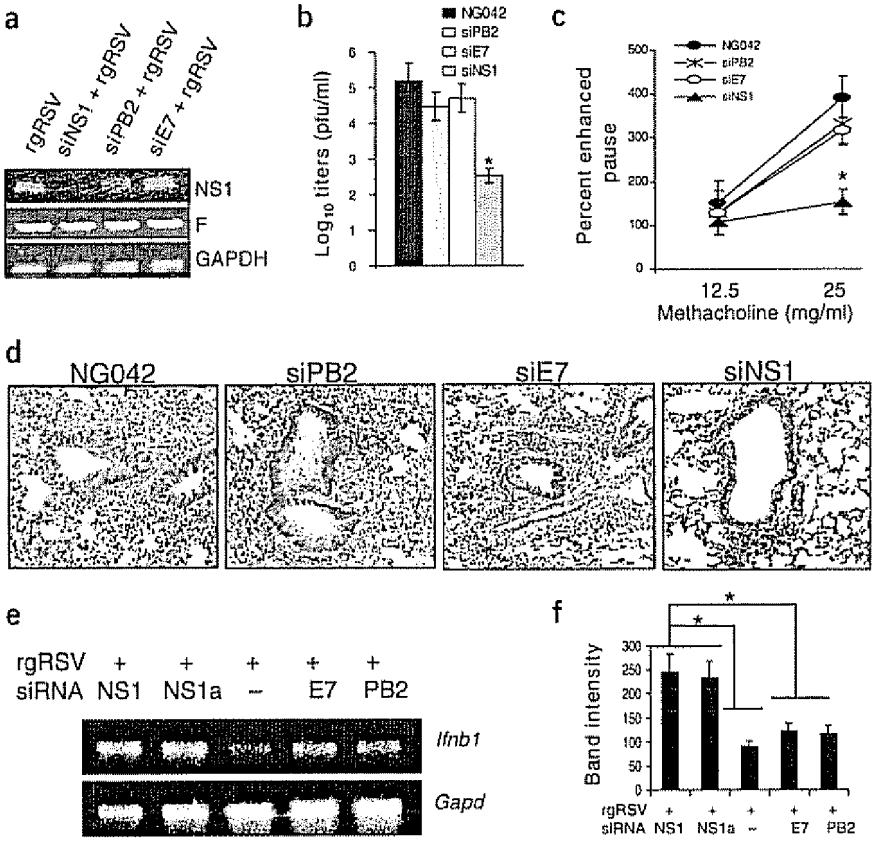

Figure 4 siNS1 exhibits antiviral activity *in vivo* (a) Detection of NS1 gene expression using RT-PCR at 18 h after infection with rgRSV (b) Determination of viral lung titer using plaque assay on A549 cells $*P < 0.05$ relative to control (c) Airway responsiveness to inhaled methacholine (MCh) was measured in mice infected with rgRSV following 2 d after prophylaxed with NG042-plasmid complex The results are expressed as percentage of Penh (enhanced pause) after inhalation of methacholine relative to phosphate-buffered saline $*P < 0.05$ compared to control (d) Histology of lung sections of mice treated as in c (staining with hematoxylin and eosin) (e) Detection of *Ifnb1* gene expression in lung tissue using RT-PCR at 24 h after infection with rgRSV (f) DNA bands were scanned using the Scion image system (US National Institutes of Health) to quantify data in e $*P < 0.05$ relative to control

or siNS1a in Vero cells. Also, in parallel studies, Vero cells cotransfected with pEGFP and siEGFP, not siNS1, showed substantial knockdown (91.68%) of EGFP gene expression (data not shown). These results show a definitive role of siNS1 and siNS1a in the attenuation of RSV replication and implicate the type-1 IFN pathway in this process.

IFNs drive a cascade of intracellular signaling, resulting in the expression of a large number of interferon-stimulated genes (ISGs) that exert the pleiotropic effects of IFN, including interference with viral replication and modulation of the host immune response[39]. The level of expression of IFN-inducible genes in infected A549 cells treated with siNS1 was considerably altered, as shown by the microarray data. IRF3 and MxA expression were upregulated after NS1 inhibition, in agreement with a previous report on bovine RSV[26], although STAT2 levels were not changed. In addition, expression of STAT1, IRF1, and ISGF-3γ, were substantially upregulated in our studies compared to control. IRF1 may have an important role in human RSV infection because it functions as a transcriptional activator[40] and binds to the positive regulatory domain I of the IFN-β promoter[41] and to the IFN-stimulated response element of IFN-stimulated genes[42]. ISGF-3γ encodes a protein-interaction function that allows recruitment of STAT1 and STAT2, their translocation from the cytoplasm to the nucleus and initiation of transcription of IFN-stimulated genes[39]. Furthermore, results show that both the IRF1 and phosphorylated STAT1 proteins translocate to the nucleus of infected A549 cells through knockdown of the NS1 protein, which suggests that NS1 targets activation of STAT1 and IRF1.

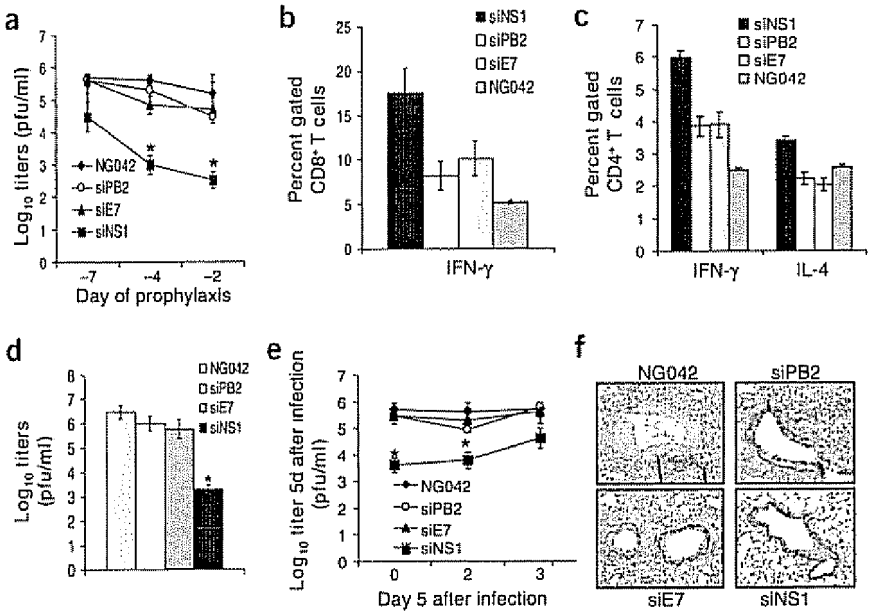

Figure 5 Prophylactic and therapeutic potential of NG042-siNS1. (a) Measurement of viral lung titer in the mice given prophylactic treatment at 2, 4 or 7 d before RSV infection using plaque assay on A549 cells. $^*P < 0.05$ relative to control. (b,c) Intracellular cytokine production in spleen T cells in the mice at 5 d after secondary infection, which were administered prophylactic treatment at day −2, inoculated with rgRSV at day 1 and day 16. (d) Measurement of viral lung titer from rechallenged mice (1×10^7 p.f.u./mouse) at day 5 after secondary infection. $^*P < 0.05$ compared to control. Results of one experiment of two representative experiments are shown. (e) Analysis of lung RSV titers at 5 d after infection by plaque assay on A549 cells of mice treated with siRNA after different days of rgRSV-inoculation as indicated. $^*P < 0.05$ relative to control. (f) Histology (staining with hematoxylin and eosin) of lung sections of mice treated with NG042-siNS1s, control siRNAs, or NG042 alone, at day 2 after infection.

An important finding of this study is that siNS1 and siNS1a induced substantially higher amounts (a threefold increase) of IFN-β compared to controls, including siE7 or siPB2 (expressed from the same plasmid vector backbone as siNS1) and the unrelated siRNA, indicating that NS1 is involved in antagonizing type-1 IFN. These results are in agreement with the increases in IFN production observed with NS1/NS2-deleted human RSV infection[25–28]. It is noteworthy, however, that compared to RSV-infected cells, cells transfected with either the vector plasmid or with siRNA targeting different viral antigens or an unrelated siRNA showed a slight increase of IFN-β production following RSV infection. This may be attributable to plasmid-driven siRNA-induced IFN-stimulated genes, including PKR and OAS[43,44], to CpG motifs (*amp*^{r} gene) present in the vector plasmid that activate innate immunity by binding to TLR9 (ref. 45), or to the U6 promoter-vector, which induces a higher frequency of interferon-stimulated genes compared to lentiviral H1 vectors[16]. The vector or control siRNA–induced IFN production also upregulates certain IFN-inducible genes, particularly those encoding STAT1 and IRF1 and IRF3, which might account for the finding that siE7 or siPB2 reduced rgRSV production by about 1 $\log_{10}$ *in vitro*. But siNS1 induces a considerably higher level of expression of these ISGs, including MxA and ISGF-3γ, and, in addition, promotes phosphorylation of STAT1.

Whereas epithelial cells are the major target cells in which the virus replicates, monocytes and DCs have a role in generating anti-RSV immunity. Monocytes have a role in the pathophysiology of RSV bronchiolitis[47], and they represent a pool of circulating precursors capable of differentiating into DCs that are able to present pathogen-derived peptides to naive T cells. NS1 seems to decrease type-1 IFN production in DCs, presumably affecting their state of activation and antigen presentation. The results of these studies show that RSV infection decreases the capacity of DCs to induce IFN-γ in naive T cells[48], which might cause the delayed RSV-specific immune response and permit multiple RSV reinfections. Thus, infected DCs treated with siNS1 produce much more type-1 IFN and also drive naive CD4$^+$ T cells toward T_H1-type lymphocytes that generate more IFN-γ and less IL-4.

The effects of siRNA have been amply shown in cultured cells. But only a few studies have addressed the potential of siRNA-based therapeutics *in vivo* using model animal systems. A notable result of this report is that a new generation of oligomeric nanometer-size chitosan particles, NG042, can be used for *de novo* expression of siNS1 in the lung tissues that results in protection from RSV infection. NG042 shows higher transduction efficiency and induces less inflammation compared to classical high molecular weight chitosan (data not shown). The results of studies on the prophylactic potential of NG042-siNS1 indicate that siNS1 induces substantial protection from rgRSV infection, infection-induced inflammation and airway reactivity, and the protective effect lasted for at least 4 d. Furthermore, even a single-dose prophylaxis with NG042-siNS1 considerably inhibits reinfection in mice that are administered a higher dose of RSV 16 d after primary infection. The precise mechanism of enhanced protection is unknown, but it is probable that knockdown of the NS1 gene augments anti-RSV host immunity through enhanced IFN production and thereby prevents

mice from RSV reinfection. In addition, NG042-siNS1 also attenuates established RSV infection. Thus, the antiviral treatment decreased viral titer in the lung, improved pulmonary function and attenuated pulmonary inflammation in rgRSV-infected mice.

In conclusion, our data show that NS1 promotes virus infection of human epithelial and dendritic cells by inhibiting the type-1 IFN pathway. Treatment with NG042-siNS1 either before or after RSV infection substantially attenuates RSV infection and infection-induced pulmonary pathology in mice. Thus, siNS1 nanoparticles may prove to be a potent, new prophylactic and/or therapeutic agent against RSV infection in humans.

METHODS

Virus and cell lines. A549, Vero cell line and RSV strain A2 were obtained from the American Type Culture Collection. Recombinant rgRSV which encodes green fluorescent protein was supplied by M. E. Peeples[31].

Plasmid constructs. The nucleotide sequence for each siRNA is as follows: siNS1: 5′-GGCAGCAATTCATTGAGTATGCTTCTCGAAATAAGCATACTCAATGAATTGCTGCCTTTTTG-3′; siNS1a: 5′-GTGTGCCCTGATAACAATATTCAAGAGATATTGTTATCAGGGCACACTT-TTTTG-3′; siE7: 5′-GAAAACGATGAAATAGATGTTCAAGAGACATCTATTTCATCGTTTTCT-TTTTT-3′; siPB2: 5′-GGCTATATTCAATATGGAAAGAACTCGAGTTTTGTTCTTTCCATATT-GAATATAGCCTTTTTG-3′; and siUR: 5′-GGTCACGATCAGAATACTTCGCTCGAGCGAAG-TATTCTGATCGTGACCCTTTTTTG-3′. Each pair of oligonucleotide sequences was inserted into pSMWZ-1 plasmid at appropriate sites respectively, to generate the corresponding siRNA for RSV NS1, HPV_{18} E7, type A Influenza virus PB2 and pUR.

DNA transfection and virus infection. Cells were transfected with siNS1 or controls (siE7, siPB2 or siUR) using Lipofectamine 2000 reagent (Invitrogen). We infected cells 24 h later with rgRSV or RSV at appropriate multiplicity of infection. We used the pEGFP plasmid (Stratagene) for measurement of transfection efficiency.

Flow cytometry. To quantify rgRSV-infected cells, cells were harvested and scored for GFP-positive cells by flow cytometry with appropriate gating and proper controls at the Moffitt Flow Cytometry Core. Additional flow cytometry analyses were conducted utilizing fluorescent-labeled antibodies.

Isolation of DCs from human peripheral blood and measurement of IFNs in supernatants of infected DCs. Monocytes purified from PBMCs using monocyte isolation Kit II (Miltenyi Biotec) were seeded into 6-well culture plates supplemented with 200 ng/ml IL-4 and 50 ng/ml GM-CSF (BD Pharmingen) and cultured for 6–7 d for plasmid transfection and infection with RSV. We assayed expression of IFNs in the supernatants by IFN-α Multi-Species ELISA Kit and IFN-β ELISA kit (PBL Biomedical Laboratories).

Analysis of intracellular cytokine production in T cells. Human naive $CD4^+$ T cells (1×10^6 cells/well) purified using $CD4^+$ T cell isolation kit (Miltenyi Biotec) from umbilical cord blood were cocultured with irradiated monocyte-derived DCs (30 Gy) (1×10^5 cells/well) in 24-well plates for 6 d with additional culture for 8 d in the presence of recombinant human IL-2 (10 ng/ml). We cultured mouse spleen T cells purified using mouse T-cell enrichment column kit (R & D Systems) in 6-well plates for 4 d. Finally, cells were stimulated with phorbol-12-myristate-13-acetate (50 ng/ml) and ionomycin (500 ng/ml) (Sigma) for 6 h in the presence of GolgiStop (PharMingen) and then fixed and stained them using CD8 or CD4 monoclonal antibody (BD Biosciences) for flow cytometry analysis.

Immunofluorescence. A549 cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 3% donkey serum in phosphate-buffered saline containing 1% glycerin for 60 min. We next incubated cells with IRF1 antibody (Santa Cruz Biotec) or phosphorylated STAT1 (Ser727; Upstate), respectively, and then with Zenon Alexa Fluor 488 (Molecular Probes). The slides were visualized by immunofluorescence microscopy.

Plaque assay. We added tenfold serial dilutions of the supernatants to a monolayer of A549 cells and replaced the medium in each well of six-well culture plates by an agarose-containing overlay (2× DMEM, 10% fetal bovine serum, 1% low melting point agarose (Gibco BRL)). The plates were incubated at 37 °C for 5 d. Afterward, we added 1% neutral red (Sigma) and counted the plaques 48 h later.

Microarray assays. Total RNAs were extracted by Qiagen RNeasy Kit. We used 10 μg of total RNAs to prepare cDNA. Gene expression was analyzed with GeneChip Human Genome U95Av2 probe array (Affymetrix) according to the manufacturer's protocol (Expression Analysis Technical Manual). We performed data analysis with Microarray Suite 5.0 (MAS 5.0).

Protein expression analysis by western blotting. Transfected A549 cells were infected with rgRSV (MOI = 1). We performed electrophoresis on the whole cell lysates using 12% polyacrylamide gels and transferred the proteins to PVDF membranes (BIO-RAD). The blot was incubated separately with RSV polyclonal antibody (AB1128, Chemicon Int.), STAT1, phoshphorylated STAT1 (Tyr701), STAT2, IRF1, IRF3, IRF7, ISGF-3γ and IFN-β (Santa Cruz), phosphorylated STAT1 (Ser727, Upstate) or MxA antibody (O. Haller). Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce).

Studies in mice. Animal studies were approved by the University of South Florida and Veterans' Affairs Hospital Institutional Animal Care and Utilization Committee. All animal studies were blinded to remove investigator bias. We administered plasmid (10 μg per mouse) with NG042 (50 μg per mouse) (TransGenex Nanobiotech Inc) intranasally to 6-week-old female BALB/c mice (Charles River, $n = 8$ per group) before or after inoculation with rgRSV (5×10^6 p.f.u./mouse). The pulmonary function was evaluated at day 4 after infection as described previously[21]. Finally, all mice were killed the next day. The RSV titer was determined by plaque assay from the lung homogenate ($n = 8$), and histological sections from lungs ($n = 8$) were stained with hematoxylin and eosin. We performed RT-PCR analysis in the lung tissue using the following primers. IFN-β: forward, 5′-ATAAGCAGCTCCAGCTCCAA-3′, reverse, 5′-CTGTCTGCTGGTGGAGTTCA-3′; RSV-NS1: forward, 5′-ATGGGGTGCAATTCATTGAG-3′, reverse, 5′-CAGGGCACACTTCACTGCT-3′; RSV-F: forward, 5′-TGCAGTGCAGTTAGCAAAGG-3′, reverse, 5′-TCTGGCTCGATTGTTTGTTG-3′; and GAPDH: forward, 5′-CCCTTCATTGACCTCAACT-3′, reverse, 5′-GACGCCAGTG-GACTCCA-3′. PCR products were visualized by gel electrophoresis and quantified by densitometry.

Statistical analysis. Pairs of groups were compared by Student's *t*-test. Differences between groups were considered significant at $P < 0.05$. Data for all measurements are expressed as means ± s.d.

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The authors declare competing financial interests (see the *Nature Medicine* website for details).

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1 Sigurs, N., Bjarnason, R., Sigurbergsson, F. & Kjellman, B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am. J. Respir. Crit. Care Med.* **161**, 1501–1507 (2000).
2 Sigurs, N., Bjarnason, R., Sigurbergsson, F., Kjellman, B. & Bjorksten, B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. *Pediatrics* **95**, 500–505 (1995).
3 Shay, D. K. *et al.* Bronchiolitis-associated hospitalizations among US children, 1980-1996. *JAMA* **282**, 1440–1446 (1999).
4 Hall, C. B., Long, C. E. & Schnabel, K. C. Respiratory syncytial virus infections in previ-

ously healthy working adults *Clin Infect Dis* **33**, 792–796 (2001)
5 Thompson, W W *et al* Mortality associated with influenza and respiratory syncytial virus in the United States *JAMA* **289**, 179–186 (2003)
6 Sly, P D & Hibbert, M E Childhood asthma following hospitalization with acute viral bronchiolitis in infancy *Pediatr Pulmonol* **7**, 153–158 (1989)
7 Brandenburg, A H , Neijens, H J & Osterhaus, A D Pathogenesis of RSV lower respiratory tract infection: implications for vaccine development *Vaccine* **19**, 2769–2782 (2001)
8 Coffin, S E & Offit, P A New vaccines against mucosal pathogens: rotavirus and respiratory syncytial virus. *Adv. Pediatr Infect Dis* **13**, 333–348 (1997)
9 Handforth, J , Friedland, J S & Sharland, M Basic epidemiology and immunopathology of RSV in children. *Paediatr. Respir. Rev.* **1**, 210–214 (2000)
10 Walliver, R C Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. *J Pediatr.* **143**, S112–S117 (2003)
11 Collins, P L , Chanock, R M. & Murphy, B R. in *Fields Virology* Vol 1, 4th edn (eds Knipe, D M , Howley, P M & Griffin, D E), 1443–1485 (Lippincott-Raven, Philadelphia, 2001)
12 Jin, H. *et al* Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2-2 genes are attenuated in vitro and in vivo *Virology* **273**, 210–208 (2000).
13 Teng, M. N. & Collins, P L Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein *J Virol* **73**, 466–473 (1999)
14 Teng, M N *et al* Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees *J Virol* **74**, 9317–9321 (2000).
15 Murphy, B.R. & Collins, P L Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics *J Clin Invest* **110**, 21-27 (2002)
16 Hall, C B , Walsh, E E , Long, C.E & Schnabel, K C. Immunity to and frequency of reinfection with respiratory syncytial virus *J. Infect Dis* **163**, 693–698 (1991).
17 Roman, M. *et al.* Respiratory syncytial virus infection in infants is associated with predominant T_H2-like response. *Am. J Respir Crit. Care Med* **156**, 190–195 (1997)
18 Matsuse, H *et al* Recurrent respiratory syncytial virus infections in allergen-sensitized mice lead to persistent airway inflammation and hyperresponsiveness *J Immunol* **164**, 6583–6592 (2000)
19 Behera, A K , Kumar, M , Lockey, R F & Mohapatra, S S Adenovirus-mediated interferon gamma gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling *Hum Gene Ther* **13**, 1697–1709 (2002)
20 Kumar, M , Behera, A K , Matsuse, H , Lockey, R F & Mohapatra, S S Intranasal IFN-γ gene transfer protects BALB/c mice against respiratory syncytial virus infection *Vaccine* **18**, 558–567 (1999)
21 Kumar, M *et al* Intranasal gene transfer by chitosan-DNA nanospheres protects BALB/C mice against acute respiratory syncytial virus infection *Hum Gene Ther* **13**, 1415–1425 (2002)
22 Kumar, M *et al* Chitosan IFN-g-pDNA nanoparticle (CIN) therapy for allergic asthma *Genetic Vaccines and Ther* **1**, 3–12 (2003)
23 Mohapatra, SS. Mucosal gene expression vaccine: a novel a vaccine strategy for respiratory syncytial virus *Pediatr Infect Dis J* **22**, S100–S103 (2003)
24 Hellermann, G., Mohapatra, SS. Genetic Therapy: on the brink of a newfuture *Genetic Vaccines and Ther* **1**, 1–3 (2003)
25 Bossert, B & Conzelmann, K K Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells *J Virol* **76**, 4287–4293 (2002)
26 Bossert, B , Marozin, S. & Conzelmann, K.K Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. *J Virol* **77**, 8661–8668 (2003)
27 Schlender, J , Bossert, B , Buchholz, U. & Conzelmann, K K Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize α/β interferon-induced antiviral response *J. Virol* **74**, 8234–8242 (2000)
28 Spann, K M , Tran, K C , Chi, B , Rabin, R.L & Collins, P.L Suppression of the induction of α, β, and γ interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages *J Virol* **78**, 4363–4369 (2004)
29 Fire, A RNA-triggered gene silencing. *Trends Genet* **15**, 358–363 (1999).
30 Zhang, W , Singam, R , Hellermann, G , Kong, X , San Juan, H , Lockey, R F , Wu, S J , Porter, K , Mohapatra, S S. Attenuation of dengue virus infection by adeno- associated virus-mediated siRNA delivery *Genetic Vaccines Ther.* **2**, 8–12 (2004)
31 Hallak, L K , Collins, P L , Knudson, W & Peeples, M E Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection *Virology* **271**, 264–275 (2000)
32 Mosca, J D. & Pitha, P M Transcriptional and posttranscriptional regulation of exogenous human β interferon gene in simian cells defective in interferon synthesis *Mol Cell Biol* **6**, 2279–2283 (1986)
33 Leaman, D W. *et al* Targeted therapy of respiratory syncytial virus in African green monkeys by intranasally administered 2-5A antisense. *Virology* **292**, 70–77 (2002)
34 Fisher, T L , Terhorst, T , Cao, X & Wagner, R W Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells *Nucleic Acids Res* **21**, 3857–3865 (1993)
35 Kole, R & Sazani, P Antisense effects in the cell nucleus: modification of splicing *Curr Opin Mol Ther* **3**, 229–234 (2001)
36 Billy, E , Brondani, V , Zhang, H , Muller, U & Filipowicz, W Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines *Proc Natl Acad Sci USA* **98**, 14428–14433 (2001).
37 Arnold, R , Humbert, B , Werchau, H , Gallati, H & Konig, W Interleukin-8, interleukin-6, and soluble tumor necrosis factor receptor type-I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus *Immunology* **82**, 126–133 (1994).
38 Atreya, P.L , Peeples, M E & Collins, P.L The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication *J Virol* **72**, 1452–1461 (1998).
39 Stark, G R., Kerr, I M , Williams, B. R , Silverman, R H. & Schreiber, R D How cells respond to interferons. *Annu Rev. Biochem* **67**, 227–264 (1998)
40 Barnes, B., Lubyova, B. & Pitha, P M On the role of IRF in host defense *J Interferon Cytokine Res* **22**, 59–71 (2002)
41 Harada, H *et al* Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes *Cell* **58**, 729–739 (1989)
42 Pine, R , Decker, T , Kessler, D S , Levy, D.E & Darnell, JE Jr. Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either *Mol Cell Biol.* **10**, 2448–2457 (1990).
43 Sledz, C.A , Holko, M , de Veer, M J , Silverman, R.H. & Williams, B.R. Activation of the interferon system by short-interfering RNAs *Nat Cell Biol* **5**, 834–839 (2003)
44 Bridge, A J , Pebernard, S., Ducraux, A , Nicoulaz, A L , & Iggo, R Induction of an interferon response by RNAi vectors in mammalian cells *Nat Genet* **34**, 263–264 (2003)
45 Sato, Y *et al.* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization *Science* **273**, 352–354 (1996).
46 Pebernard, S , & Iggo, R D Determinants of interferon-stimulated gene induction by RNAi vectors *Differentiation* **72**, 103–111 (2004)
47 Bont, L., Kavelaars, A , Heijnen, C J , van Vught, A J. & Kimpen, J L Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis *J Infect. Dis* **181**, 1772–1775 (2000)
48 Bartz, H *et al* Respiratory syncytial virus decreases the capacity of myeloid dendritic cells to induce interferon-gamma in naive T cells *Immunology* **109**, 49–57 (2003)